Development of X-ray excitable luminescent probes for scanning X-ray microscopy

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Abstract

Transmission soft X-ray microscopy is now capable of achieving resolutions that are typically 5 times better than the best-visible light microscopes. With expected improvements in zone plate optics, an additional factor of two may be realized within the next few years. Despite the high resolution now available with X-ray microscopes and the high X-ray contrast provided by biological molecules in the soft X-ray region (\( \lambda = 2-5 \) nm), molecular probes for localizing specific biological targets have been lacking. To circumvent this problem, X-ray excitable molecular probes are needed that can target unique biological features. In this paper we report our initial results on the development of lanthanide-based fluorescent probes for biological labeling. Using scanning luminescence X-ray microscopy (SLXM, Jacobsen et al., J. Microscopy 172 (1993) 121–129), we show that lanthanide organo-polychelate complexes are sufficiently bright and radiation resistant to be the basis of a new class of X-ray excitable molecular probes capable of providing at least a fivefold improvement in resolution over visible light microscopy. Lanthanide probes, able to bind 80–100 metal ions per molecule, were found to give strong luminescent signals with X-ray doses exceeding \( 10^8 \) Gy, and were used to label actin stress fibers and in vitro preparations of polymerized tubulin. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

In the past two decades, cell biology has been revolutionized by the development of highly specific fluorescent labels that can accurately target biological molecules of interest. Fluorescent probes have been used to study systems as diverse as intracellular signaling, protein processing, nuclear transcription sites, gene localization, and the structure of the cytoskeleton. Fluorescent labels with high quantum efficiencies combined with high-quality confocal systems, are routinely used to visualize biological structures with resolutions approaching the visible light limit of about 200 nm. The most direct approach to increase resolution beyond this limit is to use techniques that can...
operate at shorter wavelengths. Electron microscopy (EM) can achieve atomic resolution but requires specimen preparation and handling methods that are potentially damaging to biological materials and becomes progressively less effective for samples more than a few tenths of a micron thick. In addition, molecular labels for EM are mostly limited to derivatized gold particles that tend to suffer from problems of sparse labeling and difficulties in penetrating cells.

In recent years, soft X-ray microscopy ($\lambda = 2–5\,\text{nm}$) has achieved resolutions close to 30 nm [2]. Future improvements in zone plate lenses are expected to give resolutions approaching 10 nm. The ability of X-ray microscopes to image hydrated biological specimens up to 10 $\mu$m thick using native elemental X-ray absorption, makes it an attractive alternative to EM, eliminating the need for plastic embedding and sectioning. However, despite the improved resolution of X-ray microscopes, and the high-quality morphological detail they can provide, there has been a lack of molecular probes capable of localizing specific cell proteins and other important subcellular targets. To circumvent this problem, labeling agents are needed that are designed specifically to operate using X-ray excitation. In this regard, two approaches have been taken including dark-field X-ray microscopy using silver enhanced gold labeling as in EM [3], and scanning luminescence X-ray microscopy (SLXM, [1,4,5]). Gold labeling may prove very useful; however, it is subject to many of the same caveats as applies to its use in EM, and is further limited by the requirement for silver enhancement that makes it impractical to study multiple labels in the same specimen.

Previously, Jacobsen et al. [1] adapted the scanning transmission X-ray microscope at the National Synchrotron Light Source (NSLS, Brookhaven National Laboratory) to measure X-ray excited visible light luminescence. These workers made use of the fact that in a scanning configuration, microscope resolution is determined by the spot size of the exciting radiation rather than the wavelength of the visible luminescence. With this approach, Jacobsen and colleagues obtained a lateral resolution of about 75 nm on P31 phosphor beads, close to a three fold improvement over conventional light microscopy. Unfortunately, it was also determined that organic fluorors that have proved so useful in visible light microscopy are very labile to soft X-rays and are not stable enough to be used as X-ray probes.

In this report, we describe the first detailed results of soft X-ray biological probes based on lanthanide polychelate conjugates. We show that lanthanide probes are of sufficient brightness and stability under X-ray excitation to be used for designing high-resolution luminescent biological labels. Lanthanides were chosen for study since their luminescence is based on their atomic spectral properties and not the highly X-ray labile conjugated ring systems common to conventional fluorescent dyes. The fact that lanthanides such as Tb and Eu are common components of phosphors used in CRTs bears testimony to their ability to tolerate enormous doses of radiation. In addition, lanthanide compounds have essentially no overlap between their excitation and emission bands, i.e., they have very large Stokes shifts ($>100\,\text{nm}$). As a result, their per atom quantum yields are not significantly depressed when assembled into multimeric structures compared with single atoms [6]. This property makes it practical to increase lanthanide probe brightness by building multi-residue chelates (polychelates) capable of binding a large number of lanthanides in a single-probe molecule. In contrast, conventional organic fluorors such as fluorescein show significant self-quenching when more than a few groups are attached to probe molecules such as antibodies.

In this study we have built polychelates probes that contain 80–100 lanthanides per molecules. Photostability tests of these compounds show that lanthanide complexes continue to produce useful luminescence with radiation doses well in excess of $10^8\,\text{Gy}$. Further, luminescent yields are sufficient to provide high-quality signals with spatial resolution down to 50 nm or better. In addition to these measurements, we present the results of biological labeling experiments of actin stress fibers in fibroblasts and in vitro preparations of microtubule assemblies using polychelate conjugates of secondary antibodies, and a biotinylated polychelate that is readily adapted to avidin–biotin labeling protocols.
2. Materials and methods

2.1. Synthesis of polychelate luminescent probes

Two approaches to polychelate probe design were implemented: (a) similar to the method of Canfi et al. [6], a goat anti-rabbit secondary antibody was first linked with polylysine then acylated with the conjugate of diethylenetriamine pentaacetic acid (DTPA) and p-aminosalicylic acid (pAS), and (b) a biotinylated polychelate was made by first conjugating polylysine with biotin and rhodamine succinimidyl esters, followed by extensive substitution with DTPA-pAS. The inclusion of pAS in the chelating subunit serves two purposes. When conjugated to DTPA, pAS acts as an efficient sensitizer of Tb luminescence [7], and may contribute significantly to Tb X-ray excitation. In addition, it is a useful marker during probe synthesis and conjugation, since it facilitates measurement of Tb loading, and can be measured with a sensitivity in the picomolar range by spectrofluorometry. General preparation methods are given below.

2.1.1. DTPA-pAS monoanhydride

The monoanhydride reagent of DTPA-pAS was prepared by adding 0.714 g (2 mmol) of DTPA di-anhydride to 6 ml of anhydrous DMSO and 0.7 ml of triethylamine. To this solution, 0.356 g (2 mmol) of pAS dissolved in 3.3 ml of DMSO was added dropwise with constant stirring to give a 200 mM concentration of chelator. The mixture was allowed to react for 3 h at room temperature and used without further purification. Each reagent was obtained from Aldrich Chemicals (Milwaukee, WI).

2.1.2. Goat α-rabbit polychelate conjugate

The antibody conjugate was prepared in cooperation with Molecular Probe, Inc. (Eugene, Oregon). Goat α-rabbit IgG secondary antibody was first reacted with succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, Molecular Probes). This introduces two to three maleimide groups into the antibody for later crosslinking to polylysine. Polylysine (20–25 kD, 100–120 ε-NH₂ per chain, Sigma Chemical Co.) was substituted with thiols by reacting with N-succinimidyl-3[2-pyridyldithio] propionate (SPDP, Molecular Probes) then reduced with dithiothreitol (DTT) to generate two-to-three free sulphydryls per chain. The maleimide-antibody and polylysine-SH derivatives were then mixed and reacted overnight yielding a goat anti-rabbit polylsine conjugate. Precipitated material was removed by centrifugation. To the supernatant was added the DMSO solution of DTPA-pAS monoanhydride prepared as described above. The final DMSO concentration was kept below 20%. Aggregates were removed by centrifugation and the conjugate dialyzed against 10 mM Tris buffer pH 8.0. From estimates of the final protein content using BCA protein reagent (BioRad, CA), and the Tb binding capacity measured by spectrofluorometry (SPEX Fluorolog, SPEX Industries, NJ), each IgG bound 80 Tb per antibody.

2.1.3. Biotinylated rhodamine polychelate (BiotinRP)

To prepare the biotinylated rhodamine polychelate, 20 mg of a 20 kD polylysine (~ 100 ε-NH₂ per chain, Sigma) was dissolved in 1 ml of anhydrous DMSO (94 mM ε-NH₂). The solution was then made 5 mM in rhodamine succinimidyl ester (Molecular Probes) and biotin-LC-succinimidyl ester (Pierce Chemical, IL.) and incubated for 3 h at 37°C. The reaction mixture was then added dropwise with constant stirring to DTPA-pAS monoanhydride leaving a final fivefold molar excess of the anhydride to ε-NH₂ groups. The mixture was then incubated overnight at room temperature; any precipitate formed was removed by centrifugation. The supernatant was then dialyzed repeatedly in the dark against HBS (10 mM HEPES, 150 mM NaCl at pH 7.3) using dialysis cassettes with a 10 kD membrane cutoff (Pierce Chemicals).

Rhodamine was used in the polychelate to provide a useful fluorescent tracer to test labeling protocols using conventional fluorescence light microscopy. In addition, the chromophore provides a convenient visual marker for following the polychelate through subsequent purifications and conjugations. Fig. 1 shows the basic polychelate probe design.
2.2. Cells

NIH 3T3 mouse fibroblasts were cultured in Dulbecco modified Eagles medium containing 10% calf serum (Gibco) at 37°C with 5% CO₂. Wi38 embryonic lung fibroblasts were similarly grown except 10% fetal calf serum was used. Specimen substrates consisted of either formvar coated 200 mesh gold under grids, or 1000 Å thick silicon nitride windows (3.5 x 3.5 mm) etched in 12 mm silicon squares. Fibronectin coating was found to greatly improve cell adhesion to both grids and silicon nitride windows and was used routinely. In either case, substrates were placed in petri dishes, covered with media, then layered with cells and cultured for 1–3 d until a convenient but subconfluent density was obtained. Specimens for actin labeling were then washed with phosphate buffered saline (PBS) pH 7.4, permeabilized for 30 s with 0.5% Triton X-100 in PBS, then fixed with a solution containing 4% paraformaldehyde, 2% glutaraldehyde, 0.5% Triton X-100, 5 mM MgCl₂, 150 mM NaCl, 1 mM EGTA, and 10 mM phosphate at pH 7.4 for 1 h. Specimens were subsequently stored in PBS at 4°C until ready for labeling.

2.3. Microtubule assemblies

Monomeric tubulin was dissolved to a concentration of 1 mg/ml in buffer containing 10 μM taxol, 2 mM MgCl₂, 150 mM NaCl, 1 mM GTP, and 10 mM phosphate buffer, pH 7.4. Polymerization was allowed to proceed at 37°C for 30 min. Ten μl of the polymerized suspension was then allowed to settle for 10 min on polylysine treated formvar coated grids, wicked off with filter paper, and air dried. The dried tubules were then fixed with 4% paraformaldehyde in PBS pH 7.4 for half an hour. The fixed tubules were gently rinsed 3 times for 10 min with HBS and labeled immediately (see Labeling Protocols, below).

2.4. Labeling protocols

Total cell actin (F- and G-actin) in 3T3 cells was labeled by first incubating with a rabbit anti-actin primary (Sigma) suspended in 1% bovine serum albumin in TBS (10 mM Tris, 150 mM NaCl, pH 7.4) for 1 hr. This was followed by three 10 min rinses in 1% BSA-TBS, then treatment for 30 min with the goat anti-rabbit-polychelate-Tb antibody (50 μg/ml). After three washes in BSA-TBS, a final rinse was done with 15 mM NaCl and the specimens allowed to air dry.

To label cell F-actin with biotinRP, fixed permeabilized 3T3 specimens were incubated for 20 min with a 20:1 dilution of phallolidin–biotin (Molecular Probes) in HBS. Specimens were rinsed twice in HBS, then twice with Tris buffered Superblock (TSB, Pierce Chemicals). This was followed by incubation with streptavidin (10 μg/ml in TSB) for 30 min. Subsequently, specimens were rinsed once with TSB, three times with HBS, then treated with Tb loaded biotinRP (25 μg/ml) for 30 min. Finally, samples were rinsed 3 times in HBS and twice in distilled water before air drying.

Microtubule assemblies deposited on formvar coated EM grids were first labeled for 30 min with an anti-β tubulin mouse monoclonal (CalBiochem, CA) diluted 1:50 in TSB. After three 10 min rinses in TSB, the tubules were incubated for 30 min in biotinylated goat anti-mouse (CalBiochem, 1:50 in TSB) then rinsed three times in TSB for 10 min. This was followed by incubation with streptavidin
(10 µg/ml in TSB) for 30 min, three rinses in HBS, then a 30 min incubation with Tb loaded biotinRP. Samples were then rinsed three times with HBS, twice with water, and finally air dried.

2.5. Scanning luminescence X-ray microscopy (SLXM)

Fig. 2 shows the basic X-ray microscope configuration used in the present studies. It is essentially identical to the original design of Jacobsen et al. [1] with the exception that a higher numerical aperture objective is used for light collection and the photomultiplier has been replaced with a more quantum efficient avalanche photodiode (APD, EG&G, Canada). The APD design was described previously [5]. Taken together at least a five fold improvement in detection efficiency is expected over the earlier PMT design with significantly reduced dark noise (5–20 cps dark count rate for the APD). A zone plate lens with a 45 nm outer zone width was used throughout these experiments with a nominal Rayleigh resolution of 56 nm [1].

For X-ray photobleaching experiments, Tb loaded antibody-polychelate was deposited on a formvar coated gold EM grid and dried. The X-ray beam was then focused on the antibody-polychelate film and irradiated with 30.9 Å X-rays while the luminescence signal was simultaneously measured. This wavelength was chosen because it provides the highest available flux from the X1-A beamline. To further boost the incident X-ray flux for luminescence imaging, helium gas was used to purge the air space between the sample and zone plate-exit window assembly. X-ray transmission calculations were done using X-CAL 6 software for Macintosh (Oxford Research Group, Richmond, CA), which incorporates the X-ray data tables of Henke et al. [8].

2.6. Fluorescence light microscopy

Visible light fluorescence images were taken using an Olympus OM-2 microscope equipped with a Xillix Model 1400 cooled CCD camera. Camera operation was controlled via a Power Computing Power Center 150 equipped with a Snapper image acquisition board (Technical Instruments, CA) and IPLab Spectrum software.
3. Results

3.1. Luminescence vs. dose

An X-ray excitable probe must be able to tolerate exposure to 2–5 nm X-rays most commonly used for X-ray microscopy. X-rays of these wavelengths are in the range of the K-shell ionization energies of carbon (284 eV) and nitrogen (410 eV) so that absorption of a single photon by a chemical dye such as fluorescein is likely to destroy the conjugated aromatic rings essential to its fluorescence. This is in addition to radical damage resulting from hydroxyl and other highly reactive species generated by X-rays in the media.

To determine the radiation stability and luminescent yield of a model lanthanide probe, a comparatively thick film of a terbium loaded goat α-rabbit polychelate conjugate was deposited onto a formvar coated EM grid. Fig. 3 shows the X-ray transmission image (left) and the corresponding luminescence image (right) for this compound. Thicker regions of the film show up as dark areas in the transmission image and bright areas by luminescence. Also, various cracks can be seen throughout the dried film that are useful for calibration purposes. To evaluate the quantum yield of the probe (visible light emitted/X-ray absorbed) and its radiation stability, the X-ray excited luminescence was followed over time from the central region of the antibody film.

To estimate the absorbed dose, it is necessary to determine the thickness of the antibody probe layer. This is done by (a) measuring the flux transmitted through a region of formvar without probe, \( I_t \), and (b) through probe and formvar region, \( I_{t+p} \). Eqs. (1)–(3) are then used to estimate the thickness of the probe film:

\[
I_t = I_0 e^{-\mu_t x_t} \quad \text{(through formvar),}
\]

\[
I_{t+p} = I_0 e^{-\mu_t x_t} e^{-\mu_p x_p} \quad \text{(through probe and formvar),}
\]

\[
\frac{I_{t+p}}{I_t} = e^{-\mu_p x_p},
\]

where \( I_0 \) is the incident flux (photons cm\(^{-2}\) s\(^{-1}\)), \( \mu_t \) and \( \mu_p \) are the formvar and probe X-ray absorption coefficients (cm\(^{-1}\)), and \( x_t \) and \( x_p \) are their respective thicknesses.

From measurement of the Tb binding capacity of the antibody (80 Tb per molecule), the chemical formula for DTPA-pAS substituted polylysine, and a formula for protein, an elemental composition for the antibody conjugate of \( C_{107}O_{47}N_{30}H_{188}S_{0.2}Tb_1 \) is obtained. This gives an estimated mass absorption coefficient (\( \mu_m \)) of \( 1.3 \times 10^4 \text{ cm}^2/\text{g} \) at a wavelength of 30.9 Å [8].

![Fig. 3. X-ray transmission (left) and luminescence (right) images of terbium loaded goat α-rabbit antibody. Antibody terbium capacity equaled 80 Tb per IgG or 6% metal by weight. (Cross hairs indicate center of X-ray beam used to collect data in Fig. 4.)](image-url)
assuming a density of 1.3 g/cm³ this gives μₚ of 1.7 × 10⁶ cm⁻¹. From Eq. (3) and the measured values for Iᵣ₊ₚ and Iᵣ, an estimated thickness (xₚ) of 1.44 μm is obtained for the irradiated region marked in Fig. 3.

To get the dose delivered to the sample, the actual number of photons absorbed per unit mass in the film must be calculated. Several corrections are needed for this estimate including:

(a) the efficiency of the gas proportional counter (GPC) used for X-ray detection (66%; Kirz, personal communication);
(b) X-ray transmission loses in the air gap (2 mm) between the sample and GPC (≈ 63% transmission, λ = 30.9 Å);
(c) the use of He in the air gap between the zone plate and the sample to increase the incident flux for luminescence;
(d) the use of a larger monochromator exit slit width (400 μm versus 200 μm) for luminescence compared to the transmitted X-ray measurement.

The last two factors contribute an increase of about fourfold to the incident intensity. Correcting for all these effects gives the X-rays absorbed during luminescence imaging versus X-ray transmission as

\[ I_{\text{abs}} \approx 4I_{\text{abs}}/0.66/0.63 = I_{\text{abs}} 9.6. \]  

(4)

In theory, a 45 nm zone plate will produce a spot size of 56 nm diameter. However, since a large slit width was used (400 μm), the spot size is increased because the beam is (a) less monochromatic and (b) there is a loss of spatial coherence. The latter factor predominates. The demagnification of the exit slit width is proportional to the ratio of the distance between the source and ZP (3.1 m) and ZP to image plane (0.0013 m). Thus, a 400 μm exit slit gives a spot size about 0.168 μm. Spectral blurring and position scanning noise probably increase the spot size further. Therefore, for purposes of calculation a value of 0.2 μm diameter is used for the area of illumination. Combined with the 1.44 μm thickness estimate of the probe, the dose for a 100 msec dwell time is calculated to be 2.53 × 10⁶ Gy averaged throughout the thickness of the antibody. However, because of the thickness of the film, there is a very large degree of attenuation of the beam as it passes through the sample (92%). This means that the luminescent signal is coming predominantly from the front face of the probe film. To obtain an accurate estimate of the photobleaching parameters, the effects of attenuation are taken into account as follows.

At any depth x, a slab with an absorption coefficient μₓ receives X-ray photons with an intensity

\[ I(x) = I_0 e^{-\mu_x x}. \]  

(5)

The X-ray intensity absorbed in some thickness dx is

\[ I_{\text{abs}} = I_0 dt e^{-\mu_x x} \, dx. \]  

(6)

The dose rate in photons per unit mass per second at position x is then

\[ \text{DoseRate} = \frac{I_0 \mu_x e^{-\mu_x x}}{\rho} \, dx = I_0 \mu_m e^{-\mu_x x}, \]  

(7)

where ρ is the density and μₘ is the mass absorption coefficient (μₓ/ρ = μₘ). Eq. (7) may be converted to Gy/s by multiplying by 1.9864 × 10⁻²⁰/λ.

Bleaching can be represented as a dose-dependent change in the initial quantum yield, Q₀, (visible light photons/X-ray photon). A single exponential decay process would then be given by

\[ Q = Q_0 e^{-k t \, \text{DoseRate}}, \]  

(8)

where k is the bleach constant. Thus, for any time t and any depth x, the luminescent rate will be

\[ \text{Lum}(x, t) = Q_0 \exp[ -k t I_0 \mu_m e^{-\mu_x x}] I_0 \mu_x e^{-\mu_x x} \]  

(Q for time t at position x)

(9)

or for multiple decay processes with a non-bleachable component, Qₘ,

\[ \text{Lum}(x, t) = I_0 \mu_x e^{-\mu_x x} \times \left( \sum_i Q_i \exp[ -k_i t I_0 \mu_m e^{-\mu_x x}] + Q_0 \right). \]  

(10)

Finally, to calculate the luminescent rate as a function of time for a thick sample, we must integrate (10) with respect to the film thickness and the finite
dwell time (dwt) used for sampling so that

\[
\text{Lum Rate} (t_j) = \int_0^{\infty} \int_{(j+1)\text{dwt}} \ldots \int_{j\text{dwt}} e^{-\frac{I_0}{x}} \sum_i Q_i \exp[-k_i t I_{\text{phot}} e^{-\mu i t}] + Q_a] \, dt \, dx,
\]

where \( j = 0, 1, 2, \ldots \) \# of samples \( = 200 \).

Fig. 4 shows the bleaching of the terbium-antibody conjugate as a function of the exposure time fit to two exponential decays and a non-zero asymptote using least squares and numerical integration of (11). In terms of dose, the estimated 1/e components for bleaching are \( 3.3 \times 10^7 \) and \( 2.1 \times 10^8 \) Gy with apparent initial \( Q_i \)'s of 0.0031, 0.0027, and a \( Q_a \) of 0.004. This gives a total initial \( Q \) of 0.01. Correcting for the numerical aperture of the detector objective lens (N.A. = 0.85) and the efficiency of the avalanche photodiode (~ 50%), the estimated initial \( Q \) is 0.2 visible light photons/X-ray. Since the terbium antibody conjugate contains 6% Tb by weight, about 95% of the X-rays are absorbed by carbon, nitrogen, and oxygen, i.e., Tb captures only one X-ray out of twenty absorbed. If the terbium emission were due solely to X-ray absorption by the lanthanide, then for every X-ray photon absorbed, four visible light photons would have to be generated. It is difficult to see how single X-ray absorption events in Tb could lead to multiple visible photon emissions, suggesting that secondary electrons account for a significant fraction of the total visible light produced.

3.2. Biological samples

Fig. 5 shows some of the first scanning luminescence X-ray microscope (SLXM) biological images obtained indicating the feasibility of using the lanthanides for luminescent labeling. Fig. 5A and B shows 3T3 mouse fibroblasts labeled first with a rabbit anti-actin primary antibody followed by a goat \( \kappa \)-rabbit terbium conjugate. Using the anti-actin primary has the effect of labeling both monomeric and filamentous actin and generally produces a disperse labeling of the cytoplasm as well as microfilaments. Fig. 5C and Fig. 5D shows examples of actin labeled using a biotinylated polychelate in Wi38 fibroblasts (see Methods). In these two examples, the primary label is phalloidin–biotin, which binds very specifically to filamentous actin. After treating with the primary, the cells were incubated with streptavidin then treated with the biotinylated polychelate that binds to unoccupied valencies on the streptavidin. Again, filaments are clearly distinguishable.

Fig. 6 shows a series of images taken with increasing magnification and resolution of 3T3 cell actin filaments labeled with Tb loaded biotinRP. In these images, filaments are clearly evident. The image dwell time in each case was a 100 ms/pixel. It is also clear that with decreasing step size more and more image detail is visible. Signal intensities along labeled filaments range from 100 to 150 counts.
Fig. 5. Actin labeled samples. A and B are 3T3 mouse fibroblasts labeled with a goat α-rabbit antibody Tb-polychelate conjugate; C and D are Wi38 fetal lung fibroblasts labeled with Tb loaded BiotinRP.

above background levels. This compares quite favorably with typical confocal measurements, in which the actual number of photons used for image construction can be much less but are displayed at 256 levels. Fig. 6 also illustrates a technical artifact that has not been completely overcome with air dried polychelate labeled specimens. This is evidenced by the luminescent particulates that appear throughout the images. Using the probes rhodamine fluorescence, these particulates do not appear by light microscopy until the samples are air dried. At this juncture, we are not certain as to the cause, but it may be due in part to surface tension related damage to the labeled cytoskeletal
structures during drying. For comparison Fig. 7 shows wet actin and tubulin specimens labeled with the biotinRP by light microscopy giving the classic labeling morphology seen with conventional organic fluors. Unfortunately, initial attempts to use dehydration techniques such as critical point drying, which require serial ethanol–water steps with increasing alcohol, appear to extract the polychelate and, so far, have proved unusable.

The necessity to use dried samples, however, can ultimately be avoided using a cryogenic stage. Although X-ray imaging of labeled wet specimens can
be done in a silicon nitride wet cell, the doses required for luminescence imaging lead to severe sample damage at room temperature [9]. By comparison, early tests with cryogenic stages indicate that biological samples can tolerate doses of soft X-rays of 109 Gy or better when frozen at liquid nitrogen temperatures [10].

A further example of lanthanide probe labeling is given in Fig. 8 that shows the transmission (A) and luminescence images (B) of labeled in vitro polymerized microtubules. Using the data obtained from bleaching of the antibody polychelate film, we can estimate the Tb content per pixel in the luminescent image. For example, from the data in Figs. 4 and 2.4, 10−18 g of Tb will yield approximately 300 (±6%) detected visible light photons for a 4.7 × 107 Gy exposure. From this we can generate the lanthanide density map shown in Fig. 9. In this figure, the peak signal corresponds to 9.9 (±0.5) × 10−18 g of Tb per 0.1 μm pixel.

4. Discussion

From the X-ray photobleaching data and the estimated X-ray quantum efficiency (see Results), we can calculate the expected signal from an ideally labeled 24 nm diameter microtubule 50 nm in length covered by a 6 nm single layer of the goat antibody probe containing 6% terbium. A 2.0 × 108 Gy exposure should yield about 1030 visible light counts. This could be improved several fold using...
probes such as biotinRP that can contain \( \sim 25\% \) Tb by weight. Additional improvements would result from better light collecting optics such as an oil objective (N.A. = 1.4, \( \sim \) two fold signal increase) or by parabolic mirror designs such as that developed by Oxford Instruments (UK) for use in cathodoluminescence that have almost a full 2\( \Pi \) collection angle. Taken together, these improvements could provide nearly a 10 fold safety margin in cases where labeling is not close to ideal.

The photobleaching behavior in Fig. 4 suggests that multiple factors contribute to lanthanide excitation. There are three likely relevant mechanisms including: (a) direct excitation of the lanthanide by an \( N \) level photoelectric event, (b) direct secondary electron excitation of the lanthanide, and (c) secondary electron excitation of a nearby organic fluor (pAS) that can excite the lanthanide by intramolecular energy transfer. As indicated in Results, the latter appears to be a significant contributor to probe luminescence at low-to-moderate doses of X-rays. \( P \)-amino-salicylic acid when conjugated to DTPA-Tb is an efficient sensitizer of Tb luminescence [7]. Secondary electrons generated in the probe by the incident X-ray beam can give rise to pAS excitation that is rapidly transferred to the lanthanide. A direct hit on pAS by an X-ray, however, is likely lead to photolysis and a gradual reduction in the \( Q \) of the probe. The fact that at least two exponentials are required to fit the bleaching data suggests that even after pAS photolysis, the remaining organic material can provide secondary electrons that excite the lanthanide, but are degraded by continued X-ray exposure. The importance of pAS to probe brightness is further supported by the recent preliminary finding that inclusion of two pAS ligands per DTPA in the polychelate increases the luminescence yield several fold with X-rays (data not shown). This finding could prove very useful in the development of future polychelate probes with higher quantum efficiencies.

The fact that secondary electron excitation appears significant raises an issue regarding the limiting resolution achievable using luminescent probes. It was stated earlier that for a scanning system the resolution is determined by the spot size of the illumination. However, a more precise view is that the resolution limit is determined by the excitation volume generated by the radiation. In the case of soft X-rays, secondary electrons generated by the beam can travel some distance from where they are produced and potentially give rise to probe excitation outside the radiation cone of the focused beam. At present, there is little information about the mean free path of soft X-ray generated secondary electrons in biological materials. However, it has been estimated that absorbed X-rays of less than 500 eV deposit their energy within a range of 5–20 nm in organic solids such as polystyrene [11,12]. These values are sufficiently small that the apparent beam spread owing to secondary electrons is not likely to compromise resolution at the 20–50 nm level.

A potentially useful property of lanthanide labels such as Tb and Eu, is the comparatively long lifetimes of their excited states (100–1000 \( \mu s \), [13]). This makes it possible to eliminate essentially all unwanted sources of luminescence other than the lanthanide. Objective lenses, support films such as formvar, and bio-organic material in general, generate some background luminescence when irradiated by soft X-rays. (Background count rates in the experiments reported here were typically 400–500 cps.) However, the fluorescence lifetime of such materials will usually be under a microsecond. By suitable X-ray pulsing and acquisition delays, all but the long-lived lanthanide luminescence can be effectively blocked. This approach has been successfully used to image lanthanide labeled biological specimens with signal-to-noise ratios that are about 400 times better than comparably labeled fluorescein specimens [14]. The long lifetimes of the lanthanides, however, can be a disadvantage for rapid sample imaging. In order to preserve resolution, mixing of the afterglow from a previously illuminated spot with a newly irradiated pixel must be prevented. This can be done by imposing acquisition delays between the sampling of each pixel at the cost of longer image acquisition times.

Using different lanthanides such as Tb and Eu, which have very distinct and easily resolved emission lines, also makes it possible to do simultaneous multiple label imaging. This will be a great advantage since the high resolution provided by SLXM will be best appreciated in studies of the functional
geometry of subcellular organelles that are at present too small to resolve without resorting to thin sectioning and other preparative demands of electron microscopy. In this context, McEwen et al. [15] showed that X-ray-based 3-D tomographic reconstructions are feasible at 50 nm resolution using dose fractionation so that sample irradiation is kept within tolerable limits (\( \leq 10^9 \) Gy with cryo). The same dose fractionation principle applies for the case of luminescent labeled samples. Therefore, it should be possible to perform 3-D reconstructions on multiple probe labeled specimens without sectioning at 50 nm resolution.

Although the potential advantages of SLXM are clear, for biological application certain practical considerations must be taken into account. At present using the NSLS STXM to collect even the modest signal intensities shown in Figs. 5 and 6, and 8, very long dwell times must be used (\( \geq 100 \) ms). For a 256 \( \times \) 256 pixel image, acquisition times close to 2 h are required. Lengthy imaging times could in part or in whole be overcome by amplifying the labeling using multiple sandwiching passes of the probes or enzyme based amplification methods such as that used by de Haas, et al. [16]. However, this increases the risk of non-specific background staining and a potential loss of resolution. A better alternative is make a brighter probe with a higher quantum efficiency. As indicated earlier, initial photon yield measurements for a probe containing two pAS residues per chelate site, increase probe brightness by several times. An additional pAS residue could be substituted giving three donor pAS rings per chelate and very likely improve the brightness of the probe even further. Labeling with lanthanide loaded latex spheres is also a possibility [1]. In principle, a 20–30 nm lanthanide loaded latex sphere could bind thousands of lanthanides to a target site; however, as with gold particles of this size, good labeling can be difficult to achieve because of cell penetration and steric hindrance problems.

The alternative approach to obtaining faster acquisition times is to operate a SLXM using a brighter third generation X-ray source such as the Advanced Light Source (ALS) in Berkeley. A dedicated biological instrument at the ALS could provide high-resolution images using the current probe design in a few minutes. The ideal microscope configuration would also include a cryogenic tilt stage for full 3-D reconstruction of specimens labeled with multiple probes.

5. Conclusion

In this paper we have demonstrated that lanthanide polychelates have the requisite brightness and radiation stability to form the basis of a new class of X-ray excitable molecular probes that can take advantage of the high-resolution capability of scanning X-ray microscopes. In contrast to conventional organic dyes, lanthanide polychelate complexes were shown to yield strong luminescent signals with doses of soft X-rays exceeding \( 10^9 \) Gy. On the basis of X-ray photostability tests, calculations indicate that doses in the range of \( 10^8 \) Gy should be capable of producing specifically labeled images with signal-to-noise ratios comparable to good visible light CCD microscopes, but at 5 times better resolution. For practical application, however, brighter X-ray sources or probes with improved luminescent yields will be required for productive biological applications because of the long acquisition times necessary with currently available scanning X-ray microscopes.

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