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A low noise, high efficiency photo-avalanche diode detector and improved visible light collection optics have been adapted to the scanning transmission X-ray microscope (STXM) at the National Synchrotron Light Source (NSLS, Brookhaven National Laboratory). Using this apparatus, a new class of X-ray resistant lanthanide luminescent probes were investigated. Bleaching of lanthanide chelates during continuos irradiation indicated significant luminescence yields even after X-ray exposures greater than 10^{10} rads. By attaching lanthanide chelates to antibodies, probes specific for important cellular targets can be prepared and visualized using the finely focused X-ray beam provided by STXM (see accompanying paper by Moronne et al., for details). As shown by Jacobsen et al. (1993), the resolution of such a system is determined by the size of the area stimulated by the focused X-ray beam and not by the wavelength of the emitted visible light. 3T3 fibroblasts labeled with a rabbit anti-actin primary and then a goat anti-rabbit secondary conjugated to a Tb chelate produced luminescent staining in areas known to contain high concentrations of actin such as the nuclear periphery and lamellipodia. Details of the apparatus and example images are shown.

1. Introduction

Using efficient fluorescent probes, the best visible light microscopes are capable of spatial resolutions of 200 nm in labeled biological samples. To increase resolution beyond this limit requires techniques that can operate at shorter wavelengths. In recent years, soft X-ray microscopy has achieved

resolutions several times better than the best visible light microscopes [2]. Further, Jacobsen et al. [1] have demonstrated that visible light luminescence can be stimulated by soft X-rays, using the scanning transmission X-ray microscope (STXM) at the National Synchrotron Light Source (NSLS, Brookhaven National Laboratory). These workers obtained better than visible light resolution (about 75 nm) by raster scanning a sample across the focused X-ray beam and collecting the emitted visible light. In the final fluorescence image, the intensity value of each pixel is given by the photon counts associated with each scanned position. Importantly, the resolution is determined by the size of the focused spot of exciting radiation and not the wavelength of the emitted light. In this paper we report on improvements made to the original design of Jacobsen et al. (1993) for detection of X-ray excited visible light luminescence and present results on a new class of X-ray excitable fluorescent probes (see Moronne et al., this conference for details pertaining to the fluorescent probes).

2. Experimental Setup

STXM operates at the X-1A undulator beamline at the NSLS [4]. The undulator radiation coming from a grating monochromator is focused by a Fresnel zone plate (45 nm outer zone width) to form the X-ray microprobe. The sample is scanned in the focal plane through the X-ray spot using a high resolution piezo scanning stage. For X-ray transmission images, a gas proportional counter is used to detect the transmitted X-ray flux. For monitoring the X-ray stimulated luminescent radiation, a low noise, high efficiency silicon avalanche photodiode [5] was used for single photon counting in the range from 400-800 nm. The efficiency of the diode is expected to exceed 40% for the wavelength of the emitted luminescent radiation characteristic of lanthanide based fluorescent probes. To reduce dark noise, the photo diode is cooled to -25°C; the manufacturer claims a dark count rate of less than 100 counts/sec. In practice we measured dark count rates of 500-600 Hz. However, these rates are probably the result of light leakage in the present experimental setup. Each incoming photon produces a positive 2 volt high digital pulse output of approximately 200 ns width. These pulses are converted to a 0.7 volt NIM signal and counted using the existing data collection hardware at STXM.

The overall detection efficiency of the arrangement is given by the efficiency of the detector multiplied by the fraction of light collected behind the sample. Initially a 50x (0.6 N.A.) objective lens was used; later this was changed to a 60x (0.85 N.A.) lens that when focused on the back plane of the sample permits collection of approximately 20% of the light emitted in the full solid angle. Ignoring transmissive losses in the lenses the overall detection efficiency of the system is expected to be about 10%. An additional factor of 2.7 could be achieved using an oil objective raising the efficiency to

27%. This will be implemented in the future. The circular sensitive area of the diode has a diameter of 100 μm. This creates critical alignment demands on the light collection optics. To facilitate localization of the collected light on the active photodetector area, a second 20x demagnifying lens was used to project the signal onto the diode. The arrangement is shown schematically in figure 1.

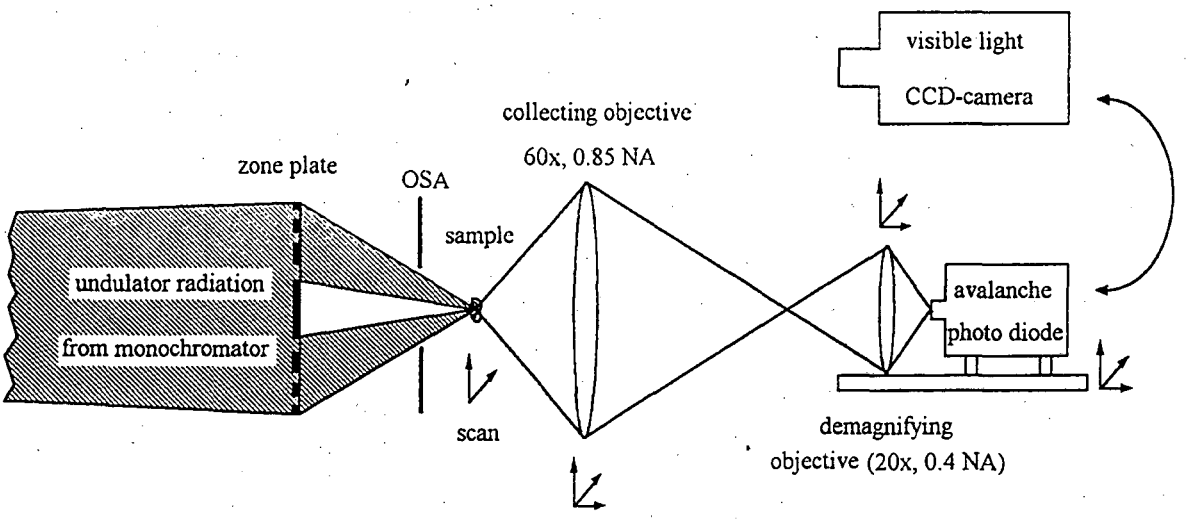

Figure 1: Experimental setup for the visible light luminescence detection at STXM. The sample is scanned through the X-ray spot. Stimulated luminescence is collected by an objective and projected by a second lens onto the sensitive area of the photo diode. To examine the sample, the diode can be replaced by a visible light CCD-camera.

In this setup the demagnifying objective and the detector are mounted together onto a holder and are adjusted with respect to each other using a standard microscope tube (160 mm) and an eyepiece in front of the lens. The holder itself is adjustable perpendicular to and along the optical axis and is aligned with respect to the collecting objective. Since it is necessary to focus the collecting objective onto the sample it is possible to remove the holder and replace it by either an eyepiece or a visible light CCD-camera. The collecting objective then works as a visible light microscope objective. The sample can be examined and the area of interest selected. The detector holder itself is mounted on kinematic mounts facilitating accurate repositioning of the detector without the need for realignment. To optimize the alignment we used P31 phosphor grains as samples. The emitted luminescent radiation from these grains is constant over time which greatly facilitates determining the optimum position of the detector. Once the system is aligned, the sample can be changed without losing positions of the objective lenses and the detector. The working distance of the collecting objective is only 0.3 mm. To accommodate the short working distance, sample holder supports were made from 100 μm thick MICA-sheets with a 2 mm diameter hole. This

configuration proved convenient for use with specimens mounted on electron microscopy grids used in the experiments reported in this paper.

3. Results on Terbium and Europium Dipicolinate Crystals

The following images show luminescent exitation by the scanning X-ray microprobe of terbium and europium chelates of dipicolinic acid. These specimens were prepared by drying approximately one µl of a 10 mM solution of the respective dipicolinate-lanthanide complex on either a formvar coated EM grid (terbium) or 1000 Å thick silicon nitride window (europium). Fig. 2a shows a scanned image of the terbium complex excited by 3.15 nm X-rays. The mainly emitted wavelength is 550 nm. This image was obtained using a long working distance 50x (0.6 N.A.) objective for light collection. When the X-ray flux of the microprobe was optimized by maximizing the exit slit of the monochromator and by flowing Helium between the sample and the zone plate lens of STXM to reduce X-ray absorption by air, peak photon count rates of 1.4 MHz were obtained. Although careful calibrations have not yet been done, the quantum yield for X-ray excitation of the terbium crystals is approximately in the range of 5 to 10 visible light photons per X-ray photon. Fig. 2b shows luminescence in the corresponding europium complex emitting light mainly around 630 nm. In this case, a set of relatively flat crystals were produced on the silicon nitride windows resulting a snowflake like pattern.

(a) (b)

Figure 2: Luminescence images of Terbium (a) and Europium (b) chelates of dipicolinic acid. Images were taken at the X-1A scanning transmission X-ray microscope.

Fig. 3 shows the decay of the luminescence signal with continued irradiation of the crystals. The drop in signal intensity with X-ray dose is well described by a double exponential decay with 1/e doses of 2 gigarads and 20 gigarads. At this point it is not clear what accounts for the two processes, but it is possible that initial damage to the dipicolinate aromatic structure could result in less efficient excitation of the lanthanide. Importantly, in contrast to conventional fluorochromes which have been shown by Jacobsen et al. to be effectively non-fluorescent via X-ray excitation except for the case of latex spheres, the lanthanides appear to retain substantial luminescence capability even after receiving more than 10 gigarad exposures.

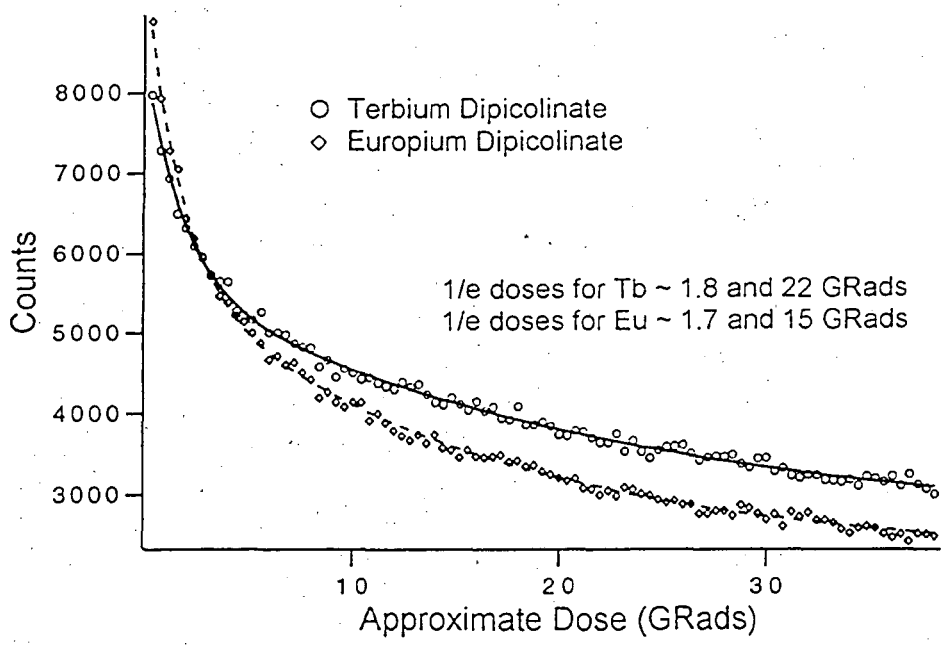

Figure 3: Decay of the luminescent signal of continously irradiated Terbium and Europium dipicolinate crystals. The dwell time per pixel is 100 ms.

4. Labeling 3T3 Mouse Fibroblasts

Fig. 4 shows a 3T3 mouse fibroblast treated with a protocol designed to target a lanthanide-antibody probe to cellular actin. The specimen was prepared by treating the fibroblasts with a rabbit anti-actin primary then followed by a goat anti-rabbit antibody conjugated to a terbium chelate. Finally the specimen was dried in air (see accompanying paper by Moronne et al. for details pertaining to specimen preparation). In the image, labeling of areas known to be rich in actin such as the nuclear periphery and lamellipodia show clear evidence of labeling. Although, the antibody probes used in these experiments are not designed to discriminate monomeric actin from filamentous actin, clear examples of actin stress fibers can be seen in the image.

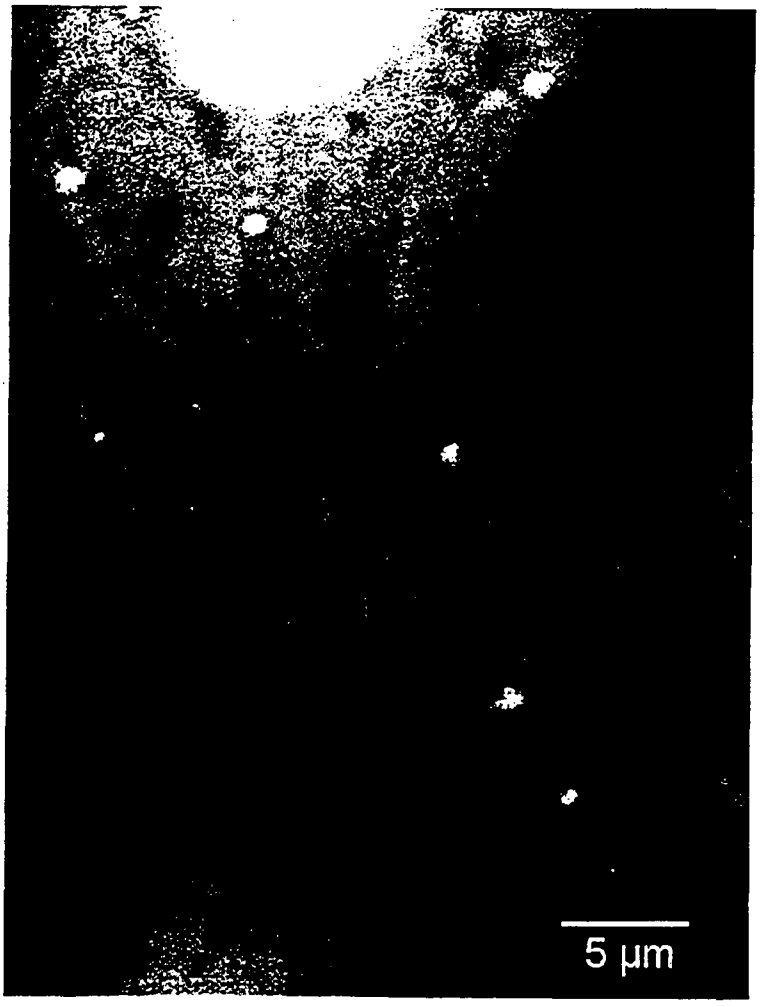

Figure 4: Luminescence image of a Terbium labeled 3T3 mouse fibroblast. The image was taken at the X-1A scanning transmission X-ray microscope with a pixel size of 0.1 μm and a dwell time per pixel of 10 ms.

5. Conclusions

With the new luminescence detection apparatus at STXM, actin filaments in 3T3 mouse fibroblasts were detected using a goat anti-rabbit terbium luminescent probe. The X-ray stability of the probe was found to be suitable for imaging cells with exposure times around 10 ms/pixel. Bleaching of the lanthanide probes was well described by a double exponential decay. However, significant luminescence was observed for doses in excess of 10 gigarads. Further improvements are expected in light collection efficiency using high numerical aperture lenses (e.g., 1.4 N.A. oil). With further developments, the technology might be able to visualize unique biological structures with far better resolution than possible with visible light methods.

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