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Visualizing specific nuclear proteins in eukaryotic cells using soft X-ray microscopy

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Abstract

Specific nuclear proteins in immunogold labeled *Drosophila melanogaster* cells were visualized by applying soft X-ray microscopy. In addition, first experiments were performed to localize two different labeled nuclear proteins in the same X-ray micrograph by using immunofluorescence microscopy for distinguishing the proteins.

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1. Introduction

Recently, it was possible to visualize 30 nm fibers in X-ray micrographs of whole, frozen-hydrated biological specimens [1]. Specific proteins, however, cannot be identified directly inside a cell. Nevertheless, since proteins carry out specific functions, often at discrete locations, mapping their distribution may help to understand their biological function. This can be accomplished by tagging these proteins with markers, e.g., using immunolabeling procedures. For example, proteins regulate dosage compensation, an essential biological process in many species. This process equalizes transcription of X-linked genes in females and males. In *D. melanogaster* this is accomplished by several proteins forming a complex on the male X chromosome, and enhancing X-linked transcription. The distribution of one of these proteins, male-specific-lethal-1 (MSL-1), in the nuclei of male *Drosophila* cell culture cells was mapped using X-ray and confocal laser scanning microscopy. It was shown that immunogold labeling and silver enhancement is a suitable method for X-ray microscopic visualization of nuclear proteins, and that about five times higher spatial resolution than in corresponding confocal laser scanning micrographs can be obtained [2].

Additional information about the function of a protein can frequently be gained by relating the

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spatial distribution of one protein to the spatial distribution of a second. In immunofluorescence microscopy this is usually done by marking the proteins with different antibodies to which fluorochromes with different spectral signatures have been conjugated. To accomplish the simultaneous localization of two different proteins in X-ray microscopy, several approaches are possible. In a scanning transmission X-ray microscope, X-ray excitable luminescent probes with different spectral emittances [3,4] could be used. In full field X-ray microscopes, antibodies conjugated to elements with absorption edges in or near the water window wavelength region, such as vanadium [5], could be used to provide protein specific contrast using differential imaging. However, these schemes necessitate the development of special probes and conjugations for X-ray microscopy.

We are investigating the possibility of solely using commercially available immunogold and immunofluorescence probes for simultaneous visualization of two different proteins in the X-ray microscope. The X-ray micrographs would provide high-resolution maps of the distribution of the two investigated proteins, and confocal laser scan micrographs would be used to distinguish the proteins. In addition to MSL-1 we investigated the distribution of Hrb57A (heterogeneous nuclear RNA binding protein at region 57A), a protein involved in the post transcriptional packaging of freshly transcribed RNA. Following indirect immunolabeling protocols, MSL-1 was tagged with both fluorophore and colloidal gold conjugated antibodies. Confocal laser scanning micrographs as well as transmission X-ray micrographs were acquired from the same samples and compared. Confocal scanning micrographs of the distribution of MSL-1 and Hrb57A in the same samples will be shown.

2. Indirect immunolabeling of MSL-1

Samples were prepared according to the following scheme: Cells of the male *D. melanogaster* cell line SL2 were grown on X-ray microscopic support foils. The samples were fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 5–7 min, then extracted for 20 min with 0.5%–1.0% TX-100 in PBS. Samples were incubated for 30 min at 37°C with the primary antibody (rabbit anti-MSL-1), washed several times, incubated for 8–12 h at 6–10°C with the 1 nm colloidal gold conjugated secondary antibody washed, and further incubated for 2–8 h with the fluorochrome conjugated secondary antibody. After this, confocal laser scanning micrographs of these samples were acquired with a LSM 510 (Carl Zeiss, Jena) equipped with an 100×/1.3 NA oil immersion objective.

While the 1 nm colloidal gold used as markers for the X-ray microscope allows a very good penetration of the conjugated antibodies into the specimen, it is too small to be visualized directly in the TXM. Therefore, it is necessary to increase the size and absorption of the markers with silver enhancement. After acquiring the confocal micrographs, the samples are fixed further with 2% glutaraldehyde, washed in deionized water, silver enhanced for ~25 min, and washed again in deionized water. The water layer covering the cells was adjusted to about 10 μm, and the object holder with the cells was plunged into liquid ethane at −170°C. Samples were stored in liquid nitrogen and transfered to the Göttingen cryo X-ray microscope at the storage ring facility BESSY I in Berlin.

Micrographs of a *D. melanogaster* cell in which MSL-1 protein was labeled are shown in Fig. 1. In

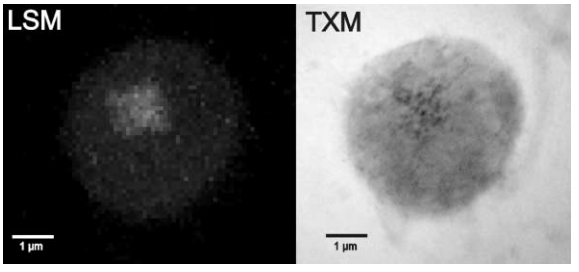

Fig. 1. Micrographs of the same MSL-1 labeled cell in the confocal laser scanning (LSM) and cryo X-ray microscope (TXM). In the LSM the fluorescent label appears as a bright region over a dark background, in the TXM the silver enhanced gold label appears as a strongly X-ray absorbing, therefore dark, region. As expected both regions appear at the same position in the specimen, the X-ray micrographs shows the substructure with better resolution.

the confocal laser scanning micrograph on the left, the brightly fluorescing areas represent the label. On the right, the same cell with the same orientation is shown in an X-ray micrograph. Here, the labeled structures appear dark, due to the strong X-ray absorption of the silver enhanced gold spheres. In both micrographs, the same regions appear labeled. Only in the X-ray micrograph is the substructure of the labeled region resolved. Smallest visible structures in the X-ray micrograph are about 33 nm, in the confocal micrograph about 180 nm in size.

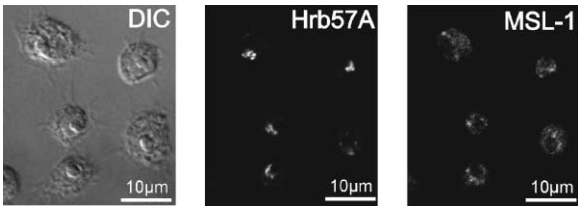

Fig. 2. These confocal scanning micrographs show several *D. melanogaster* cells. In the left image, the sample region is shown in differential interference contrast (DIC). The two other images show the same sample region, but were acquired in fluorescence mode, so that they map the distribution of the nuclear proteins MSL-1 and Hrb57A.

3. Simultaneous visualization of MSL-1 and Hrb57A

Labeling of these two proteins simultaneously, is based on the protocol described above. In the incubation step with the primary antibody, a second primary antibody, raised in mouse and directed against the second protein, Hrb57A, is added. During the incubation with gold conjugated goat-anti-rabbit antibody which detects the anti-MSL-1 antibody, a goat-anti-mouse antibody against the anti-Hrb57A antibody is added. Then the samples are incubated with fluorochrome conjugated secondary antibodies, with green fluorescent Cy2 conjugated goat-anti-rabbit, and red fluorescent Cy3 conjugated goat-anti-mouse. Fig. 2 shows confocal laser scanning micrographs of a sample region with several *D. melanogaster* cells. The left image shows the sample region in differential interference contrast (DIC). The other two micrographs show the same region in fluorescence mode, so that the spatial distribution of Cy2 and Cy3, and thus the labeled proteins MSL-1 and Hrb57A are mapped.

4. Discussion and outlook

The results reported here demonstrate the feasibility of simultaneous labeling for fluorescence and X-ray microscopy, and that micrographs from the same cells can be obtained with both instruments and compared. We also show that the simultaneous visualization of the two proteins MSL-1 and Hrb57A can be done on the light microscopic level, and that the preparation protocol is suitable for visualization of these proteins in an X-ray microscope. We plan to investigate double labeled samples with the X-ray microscope, and use LSM micrographs of the sample to distinguish the two proteins, so that high resolution maps of the distribution of two labeled proteins in whole cells can be acquired.

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