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Organelle Distribution in a Hydrated Bio-cell by Correlation between Soft X-ray and Fluorescence Images

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Abstract. Fine structures of bio-specimens obtained by soft X-ray (SX) imaging are clearer compared with those obtained by visible imaging owing to the difference in extinction coefficients between the two wavelength regions. However, it is difficult to identify the fine structure imaged in the 2.3 – 4.5 nm wavelength region by appearance alone. Here, we obtain and compare SX and fluorescence images of Leydig cells of a mouse testis loaded with fluorophores. Identification of the fine structures in the SX image is carried out by comparison with the fluorescence images with the use of principal component analysis (PCA). The result shows that the common structures between the SX- and the fluorescence- images.

1. Introduction

The soft X-rays between carbon and oxygen K-edges (λ = 2.3 – 4.5 nm) are less absorbed by water and are more absorbed by carbon, nitrogen and other elements of biological specimens [1]. When bio-cells are imaged with an SX microscope in this wavelength region, the appearance of the small organelles of the bio-cells in the images depends on both the spatial resolution of the SX microscope and absorption coefficients of the organelles. Mouse 3T3 cells have been observed by a SX microscope using a zone plate as the objective lens. Many organelles, such as nuclei and nucleoli in the nucleus, can be observed in an SX image [2]. As the nuclei and nucleoli are relatively large organelles in bio-cells, they can be easily distinguished from the other organelles. Generally, the relative sizes or characteristic shapes of organelles are not reliable markers to identify organelles in bio-cells, therefore the identification of small organelles in SX images is difficult [2].

One method to identify organelles in bio-cells is by the fluorescence labeling technique, which is generally used in biological observation to enhance the contrast of specific organelles in bio-cells. Organelles labeled with fluorophores can be identified the various organelles in a same bio-cell [3]. By the use of the fluorophores, correlative light-electron microscopy (CLEM) was applied to observe the fine structures and also to identify the locations of organelles in the bio-cells. In this method, after loading the bio-cells with a fluorophore, the locations of the organelles are obtained by fluorescence microscopy and the fine structures of the organelles are observed by electron microscopy at the exact positions of the organelle [4].
Correlative microscopy, by using an optical fluorescence microscope and SX microscope operating in the 2.3 – 4.5 nm wavelength region, will enable the observation of hydrated specimens composed of C, N and O atoms. Hence, one of the authors obtained fluorescence and SX images of Leydig cells of a mouse testis as represented in Figs. 1 [5]. The complex structures of the cellular organelles observed in the SX images were visually compared with the images obtained by fluorescence microscopy. The direct comparison between the SX and the fluorescence images showed that some structures in the SX image were identical to those observed in the fluorescence image, which suggested that the structures observed in the SX image correspond to mitochondria (MitoTracker binds well to mitochondria).

In this study, fine structures of hydrated biological specimens in an SX image, which are generally not usually identified, are identified by numerical comparison with fluorescence images that are obtained by observing the same biological specimens with a fluorescence microscope. The fine structures identified by the numerical comparison can be shown as a fine structure map.

2. Material and Methods

2.1. Experimental conditions

Poly methyl methacrylate (PMMA), with a thickness of 500 nm, was coated onto a thin glass plate and used as the X-ray photoresist film. Leydig cells from a mouse testis were placed onto the PMMA film and were cultivated for two to three days to establish good contact onto the film. The Leydig cells were loaded with MitoTracker, phalloidin and DAPI. The Leydig cells were covered with a silicon nitride (Si₃N₄) membrane with a thickness of 200 nm and enclosed into a sample holder designed to protect the bio-cells from the vacuum. The distance between the membrane and the glass substrate was controlled to be 5 μm thick by a spacer deposited on the membrane. Fluorescence images of the Leydig cells in the sample holder were observed at first by the fluorescence microscope (Eclipse Ti-U, Nikon Instruments Inc.), and then the sample holder was placed in the vacuum chamber for SX imaging. The time interval between the fluorescence- and the SX-imaging was typically 10 minutes. The spatial resolution of the fluorescence image depends on the emission wavelength of the fluorophore and was estimated to be less than 0.8 μm.

Soft X-ray images were obtained by the contact method with the use of a laser-produced plasma (LPP) light source. The X-ray pulse used for the SX imaging was generated by the high power Nd:glass laser system with the output energy of 20 J and a 600 ps pulse duration. The exposure time, which is determined by the pulse duration of the SX, is approximately 600 ps. The spatial resolution of the SX imaging was measured to be approximately 90 nm.

After exposure to soft X-rays, the PMMA was rinsed with sodium hypochlorite (NaClO) to remove the cells and was carefully observed by differential interference microscopy to make sure that no cells remained on the surface. The PMMA photoresist was then developed with a mixed solution of methyl isobutyl ketone (MIBK) and isopropyl alcohol (IPA) and the printed SX images on the surface of the PMMA were read by using an atomic force microscope.

![Figure 1](image-url) Figure 1: Experimental imaging results of Leydig cells. SX image (a), fluorescence images with MitoTracker staining (b), with phalloidin staining (c), and with DAPI staining.
2.2. Application conditions for the PCA method

Principal component analysis (PCA) is a statistical procedure that converts a set of observations of possibly correlated variables into a set of values that are linearly uncorrelated variables by the use of an orthogonal transformation. As the PCA method is applied to a pair of images under a specific condition, two eigenimages are obtained. The first eigenimage represents the average of the two original images and the other represents the common structures of the two original images [6].

Image multiplication is usually used to evaluate common structures between images under the assumption that both the signal intensities and the signal dispersions of both images are roughly equal. Before applying the PCA method to the experimental images, image multiplication was applied to analyse the characteristic structures between the images. Even after the image multiplication, some common structures between the images were not identified because of the weak signal intensity of the images, which suggests we needed to try another method to analyse the experimental images.

For the investigation of the application conditions of the PCA method, an image of 300×300 pixels was expected to be a high spatial resolution image, and a low spatial resolution image was also obtained by blurring the high spatial resolution image by using a 2D Gaussian function. After several trials, the application procedure for processing the images was determined as follows. The values of the pixels were normalized to a 32-bit grayscale so that the maximum and minimum values of the pixels were 1 and 0, respectively. After the PCA analysis was applied to the pair of images [7], eigenimages were also normalized to a 32-bit grayscale by the maximum and minimum values. The first eigenimage is the same as the average image which was scaled by a grayscale normalized with maximum and minimum values. The second eigenimage reflects the common structures between the two original images, which are easily recognizable from the original images.

2.3. Position alignment of experimental images

In many SX microscopy systems, the fluorescence and SX images are obtained separately through the use of different imaging devices. Therefore, the images obtained will be different in the position of specimen, pixel size, field of view and spatial resolution. Before applying the PCA method to the experimentally obtained images, all the differentiations, except for the spatial resolution, should be adjusted between the images.

First, the alignment of an image was performed by overlapping it with another image. The accuracy of the alignment was obtained by applying the PCA method to the selected images then changing the position of the images. The simulation result suggests that the accuracy of the alignment is limited by the worst value of the spatial resolution, and that the position of the images coincide with each other within this value. Attempting to compare the two images of the same objects with each other at different wavelengths provided an easy and reliable method to check whether the structures observed in each image were correlated or not by visible inspection. Rough alignment of the images was performed by visible inspection by finding the same structures observed in each image, such as

![Figure 2: Second eigenimages between the SX image and the fluorescence ones of MitoTracker staining (a), of Phalloidin staining (b), and of DAPI staining (c).](image-url)
some large organelles in bio-cells, and using them as landmarks in the images. The landmarks of the image were then aligned roughly to overlap one another.

The next step was to adjust the pixel sizes of the images to fit each other using the bilinear interpolation method [8]. In this method, the image with a smaller pixel size was enlarged to have the same pixel size as the other image whilst maintaining the two-dimensional intensity-distribution of the images.

The final step was to align the images numerically. In our experience, the best numerical method is largely dependent on the combination of selected images. The best results for aligning images in this study were achieved by transforming the images to be in the spatial frequency domain by the 2D-FFT method, and then the normalized correlation coefficients were obtained among the transformed images, and the position of the image was aligned to maximize the normalized correlation coefficients [9]. The images in the spatial frequency domain were retransformed to be in the spatial domain, and this was confirmed that the agreement between the images was within the accuracy criterion of the alignment suggested in Section 3. Output images with the best alignment showed subpixel accuracy.

3. Results & Discussion

Image processing, as described in Sections 2.2 and 2.3, was applied to the experimentally obtained images of Leydig cells as designated in Figs. 1 [5], and eigenimages were obtained as the result of the applications. The resulting second eigenimages are shown in Fig. 2(a), (b) and (c), which show the common structures between the SX image and the fluorescence images obtained with MitoTracker, with Phalloidin and with DAPI, respectively. The images are normalized by the minimum and maximum values of pixels and displayed by a 32-bit grayscale. Structures that were blurred in the original fluorescence images are clearly observed, especially in Fig. 2(b).

As an index for the fine structures observed in the images, the power spectra of the images are calculated. The power spectrum of a cyclically shifted signal is identical to that of the original signal. In other words, the power spectrum of a discrete periodic signal of a certain length shows no change by some offset of the signal. Therefore, the amount of fine structures in an image can be compared with that in the other image by the power spectra obtained from the images [10].

Power spectra of both the fluorescence image obtained with MitoTracker staining and the SX image are represented in Fig. 3(a), and those of both the fluorescence image obtained with Phalloidin staining and the SX image, in Fig. 3(b). The spectra are normalized at the intensity of the lowest frequency and an eigenimage is compared with the corresponding fluorescence image. The results show that the power spectrum of the eigenimage obtained from the fluorescence image obtained with MitoTracker is the same as that of the fluorescence image, and that the power spectrum of the eigenimage obtained from the fluorescence image obtained with Phalloidin in the high frequency region is higher than that of the fluorescence image and lower than that of the SX image. In

![Figure 3: Power spectra of original images and second eigenimages. The results corresponding to the MitoTracker-fluorescence images (a), and to the Phalloidin-fluorescence images (b). The result corresponding to the DAPI-fluorescence images show the same tendency as in the case of Phalloidin.](image-url)
the case of the images stained with DAPI, the power spectra show the same tendency as in the case of Mito Tracker.

The fluorescence images of the Leydig cells stained by Phalloidin, Mito Tracker, and DAPI reflect the distribution of the cytoskeleton, Mitochondria, and cell nucleus, respectively. The present fluorescence images show that the Leydig cells spread out widely and the Mitochondria exist around the cell nucleus. These results coincide well with that Leydig cells have a tendency to spread out with the central focus on its cell nucleus. As the result of the spreading, the thickness of the Leydig cells decrease with an increase of the distance from the cell nucleus. In addition, transmittance of the SX light increases as the decrease of the thickness of the cells. Therefore, the structures of the second eigenimages in the thick part of the cells show the same shapes of the fluorescence images, as designated in Fig. 2(a) and (c). Small structures that appeared in Fig. 2(b) will be originated from the cytoskeleton.

4. Summary

Four pairs of images, comprising the fluorescence and SX images, were analysed by the PCA method, and common structures were found in all eigenimages. The analytical results suggest that correlations between the structures depend on the thickness of the cells. The common structures located at around the cell nucleus show the same structures of the fluorescence images, and the structures located at the edge of the cells will be originated from the cytoskeleton.

Although the images were analysed by the present method with no specific assumptions before the analysis, common structures between the two images could be extracted without visible inspection. This fact suggests that the present method can be applied to any pairs of images composed of visible and SX images. Correlations between structures in different images will be applicable for analysing microscopic images of complex systems in the SX wavelength region.

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