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A fluorescence scanning electron microscope

Takaaki Kanemaru [a], Kazuho Hirata [b,*], Shin-ichi Takasu [c], Shin-ichiro Isobe [d], Keiji Mizuki [e], Shuntaro Mataka [f], Kei-ichiro Nakamura [g]

[a] Morphology and Core Unit, Kyushu University Hospital, Kyushu, Japan
[b] Department of Anatomy and Cell Biology, Graduate School of Medical Sciences, Kyushu University, Higashi-ku, Maidashi 3-1-1, Fukuoka 812-8582, Japan
[c] Advanced Technology Division, JEOL Ltd., Tokyo, Japan
[d] Department of Applied Chemistry and Biochemistry, Faculty of Engineering, Kyushu Sangyo University, Fukuoka, Japan
[e] Department of Nanoscience, Faculty of Engineering, Sojo University, Kumamoto, Japan
[f] International Science Technology Co., Ltd, Kasuga Laboratory, Fukuoka, Japan
[g] Department of Anatomy, Kurume University School of Medicine, Kurume, Japan

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ABSTRACT

Fluorescence techniques are widely used in biological research to examine molecular localization, while electron microscopy can provide unique ultrastructural information. To date, correlative images from both fluorescence and electron microscopy have been obtained separately using two different instruments, i.e. a fluorescence microscope (FM) and an electron microscope (EM). In the current study, a scanning electron microscope (SEM) (JEOL JXA8600 M) was combined with a fluorescence digital camera microscope unit and this hybrid instrument was named a fluorescence SEM (FL-SEM). In the labeling of FL-SEM samples, both Fluolid, which is an organic EL dye, and Alexa Fluor, were employed. We successfully demonstrated that the FL-SEM is a simple and practical tool for correlative fluorescence and electron microscopy.

1. Introduction

Fluorescence microscopy has become an indispensable microscopy technique for the examination of biological specimens, because it allows selective and specific detection of molecules at small concentrations with a good signal-to-background ratio [1,2]. It even allows one to work with intact samples, including living cells, and to see samples with the naked eye; these advantages are not available with other methods, such as electron microscopy [3]. Furthermore, recent developments in fluorescence imaging techniques have enabled the well-known Abbe barrier of about 200 nm lateral resolution to be crossed, that is, the diffraction limit for an optical microscope has approached the level of 100 nm as with 3D structural illumination microscopy (3D-SIM) [4] or even less than 100 nm, as with other techniques such as 4Pi, simulated emission depletion (STED) and photoactivated localization (PALM) [3,5].

In order to obtain a stable image with a higher resolution, however, an electron microscope is still required. Correlative study using both fluorescence and electron microscopy is usually employed to obtain substantial information on molecular localization. Fluorescent probes, which label an identical region of a single specimen not only for fluorescence microscopy but also for electron microscopy [6], such as FluoroNanogold [7], and more recently ReAsH, which is used in the tetracystein–biarsenical system [8] and small nanocrystals (Quantum dots; QDs) [9], have all been being utilized; nevertheless, complications during the specimen preparation process and during imaging with both types of microscopy are unavoidable.

One of the ways in which the complex methods for correlative microscopy could be improved would be to incorporate one microscope into another. The technology of the scanning electron microscope (SEM) is now well advanced and the image resolution of the SEM approaches that of the transmission electron microscope (TEM) [10]. It is clear that for the majority of biological samples, it is easier and less time-consuming to prepare samples for scanning electron microscopy than for transmission electron microscopy. Furthermore, the structure of the SEM makes it easier and more cost-effective to incorporate other devices into it. This led us to attempt to make a hybrid "FL-SEM" instrument in which an SEM is combined with an FM. In parallel with its set-up, some fluorophores were tested for the labeling of biological samples using the new instrument. We found that an organic EL fluorophore named Fluolid (Pub. no. US 7015002) [11], which has a high physical stability was suitable for this purpose.

The design of the FL-SEM is described and the first FL-SEM images are presented.

* Corresponding author. Tel.: +81 92 642 6048; fax: +81 92 642 6050.
E-mail address: hirata@anat1.med.kyushu-u.ac.jp (K. Hirata).

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Some of our findings have been previously reported in abstract form [12].

2. Experimental

2.1. Assembly of the FL-SEM

An SEM for wavelength dispersive spectrometry (WDS) (JEOL JXA8600 M, JEOL, Tokyo Japan) was reconstructed to create a dual-mode microscope, named the FL-SEM, which is capable of obtaining both FM and SEM images without requiring to move the specimen (Fig. 1A and B). All the parts of the built-in optical microscope unit in the SEM for WDS, with the exception of a mirror and the Cassegrain type (non-chromatic aberration type) 45 ×, NA 0.41 optical objective lens, both of which are placed within the column and have a small hole for the electron beam to pass through, were replaced with an FM fitted with a digital camera unit that was assembled in our laboratory. The unit consisted of a laser light source from an external unit (473 nm, Showa Optronics, Tokyo, Japan) (**a** in Fig. 1A and B), an emission filter (515 nm LP), an adaptor device for concentrating the laser light (**b** in Fig. 1A and B), a unit consisting of mirrors and prisms (**c** in Fig. 1A and B), an external CCD camera (Bitran Corporation, Saitama, Japan) adapted via a C-mount adaptor (**d** in Fig. 1A and B), and an eye piece (**e** in Fig. 1A and B). In the FL-SEM, an electron beam passes through small holes at the center of a mirror and the object lens in order to reach the specimen. The SEM image is then sent to an AD converter (SemAfor, JEOL SA20) via a photomultiplier (PMT). On the other hand, the excitation beam from the external unit reaches the specimen along the same passage as the electron beam within the column. The fluorescence emission from specimens is then directed to an external CCD camera. In practice, the digital image from the FM is acquired first, with the image from the SEM being sequentially acquired, in consideration of possible fluorescence damage which may be caused by the electron beam. Each of the FM and SEM images is separately displayed on a single PC screen through the CCD camera and the AD converter, respectively (Fig. 1C). Both images are then manually merged using Adobe Photoshop CS.

2.2. Animals

Under deep anesthesia induced by diethyl ether, adult male Wistar rats were perfused intracardially with PBS, followed by a mixture of 2.8% paraformaldehyde, 0.2% picric acid and 0.06% glutaraldehyde in 0.1 M phosphate buffer (PB) at pH 7.4. The diaphragm and the kidney were removed and postfixed with 4% paraformaldehyde in 0.1 M PB.

These experiments were reviewed by the Committee on Ethics for Animal Experiments of the Faculty of Medicine, Kyushu University and were carried out according to the guidelines for Animal Experiments of the University, and Law no. 105 and Notification no. 6 of the Japanese Government.

2.3. Exploitation of a new probe for FL-SEM

In a preliminary study, we attempted to use some tissues that had been labeled with GFP or FITC as FL-SEM samples. These fluorophores were almost bleached out in the process of dehydration. For this reason, we employed a new probe, Fluolid, which is a small organic fluorophore originally synthesized as an organic EL dye in our laboratory (Pub. no. US 7015002), [11]. The Fluolid dye has a high physical stability and a large Stokes shift and it shows strong fluorescence intensity in its solid state. However, it has never been applied to the labeling of biological samples. In order to test its viability, a kidney was immersed in 20% sucrose in PBS and 10-μm-thick frozen sections of the kidney were made for peanut agglutinin (PNA) staining, in accordance

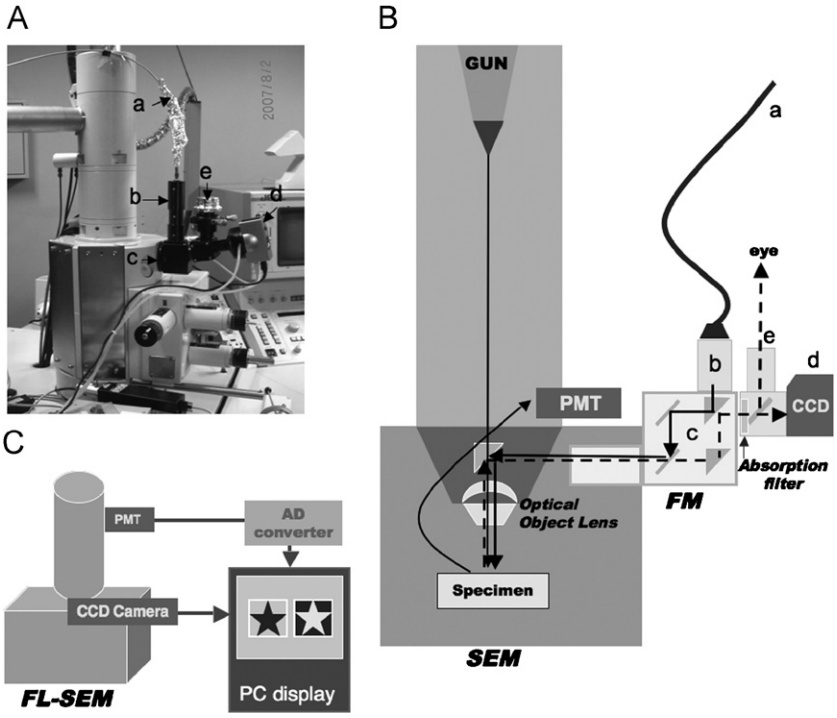

Fig. 1. (A). A view of the FL-SEM. (B). A schematic diagram of an inside view of the FL-SEM, which is made up of a combination of the SEM and the FM units. (C). A schematic diagram depicting the flow of SEM and FM image signals to a PC display. (a): the laser light source of the external unit (473 nm), (b): an adaptor device for laser light, (c): mirror and prism, (d): external CCD camera, and (e): eye piece. PMT, photomultiplier tube.