Journal of Structural Biology 185 (2014) 107–115

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Hybrid fluorescence and electron cryo-microscopy for simultaneous electron and photon imaging

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ARTICLE INFO

Article history:
Received 25 April 2013
Received in revised form 28 October 2013
Accepted 31 October 2013
Available online 7 November 2013

Keywords:
Transmission electron microscope
Fluorescence microscope
Cryo-microscopy
Correlative microscopy
Cathodoluminescence
Fluorescent protein

ABSTRACT

Integration of fluorescence light and transmission electron microscopy into the same device would represent an important advance in correlative microscopy, which traditionally involves two separate microscopes for imaging. To achieve such integration, the primary technical challenge that must be solved regards how to arrange two objective lenses used for light and electron microscopy in such a manner that they can properly focus on a single specimen. To address this issue, both lateral displacement of the specimen between two lenses and specimen rotation have been proposed. Such movement of the specimen allows sequential collection of two kinds of microscopic images of a single target, but prevents simultaneous imaging. This shortcoming has been made up by using a simple optical device, a reflection mirror. Here, we present an approach toward the versatile integration of fluorescence and electron microscopy for simultaneous imaging. The potential of simultaneous hybrid microscopy was demonstrated by fluorescence and electron sequential imaging of a fluorescent protein expressed in cells and cathodoluminescence imaging of fluorescent beads.

1. Introduction

Transmission electron microscopy (TEM) is superior to light microscopy in spatial resolution, but its field of view is limited. By contrast, fluorescence light microscopy (FLM) is superior in the field of view and selective imaging of labeled features in the specimen, but its spatial resolution is limited. Both limitations have motivated researchers to develop so-called CLEM (correlative light and electron microscopy), wherein the two imaging techniques of FLM and TEM are correlated (Caplan et al., 2011). In CLEM, FLM is employed to identify and locate features of interest, while TEM is used to map their ultrastructure (Mironov et al., 2000; Gaietta et al., 2002; Takizawa and Robinson, 2006; Schwarz and Humbel, 2007; Sartori et al., 2007; Verkade, 2008; Van Rijnsoever et al., 2008; Plitzko et al., 2009; Brown and Verkade, 2010). Traditionally, CLEM is performed using two separate setups.

Experimentation using two separate instruments has created a new problem, namely, how to colocalize a region of interest between two images obtained separately by FLM and TEM. Due to an enormous difference in the fields of view of the two microscopic techniques, the colocalization procedure is usually time-consuming and prone to errors, even when labels identifiable with both techniques can be employed. It is essential to track and conserve the coordinates of the area of interest with a high precision during the whole procedure. To avoid pitfalls and successfully exploit the potential of CLEM, integration of a standard TEM and a fluorescence light microscope has recently been proposed (Agronskaia et al., 2008; Faas et al., 2013). In this method, which has been demonstrated to achieve efficient CLEM, FLM is used to navigate to regions of interest on the TEM grids. In order to allow two different microscopy lenses to focus on a single specimen, the specimen is rotated. This is a simple and practical way to achieve a compromise between the two objective lenses, but it has a drawback: two images must be collected in a sequential manner after specimen relocation.

To fix this drawback and perform CLEM via simultaneous hybrid imaging, we have developed a novel instrument for versatile integration of FLM and TEM. In order to convert the FLM and TEM optical axes into a coaxial arrangement, our design adopted a simple reflection mirror inserted into the pole piece gap of the TEM. This design recalls the parabolic mirror used in SEM-CL, namely in scanning electron microscopy (SEM) for cathodoluminescence (CL)

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http://dx.doi.org/10.1016/j.jsb.2013.10.018

(Barnett et al., 1975; Itoh et al., 1986; Ning et al., 1992; Niitsuma et al., 2005; Fisher et al., 2008; Glenn et al., 2012) but the two mirrors have different functions as the reflection mirror is used to irradiate light to and collect light from specimens while the parabolic mirror used in SEM-CL only to collect light emitted from specimens under scanning of electron beam. Although the hybrid microscope was originally designed to manage two operations of FLM and TEM sequentially for the same specimen, it was later found that this kind of design could also manage a simultaneous imaging of FLM and TEM for a target and moreover the combination of electron irradiation and light detection enabled a noble kind of microscopic imaging, namely a genuine TEM-CL imaging without electron scanning. Conventionally CL imaging has been conducted with SEM, where the CL emission from specimens is collected through the parabolic mirror to generate a scanning image. Though CL images generated with TEM were once claimed (Yamamoto, 2002; Strunk et al., 2006), what was actually done was a CL imaging with the scanning mode of STEM (STEM-CL). The type of CL imaging shown in this paper must be the first one ever reported.

The hybrid microscope proposed in this report has been tested and characterized by using fluorescent beads and a cell system having actin filaments which are genetically tagged with a fluorescent protein.

2. Materials and methods

2.1. Materials and plunge-freezing method

Gold TEM grids (Au NH_2 finder grids R2/2 or Au 200 mesh grids R1/4; Quantifoil Micro Tools) were used as a substrate for PtK2 β-actin-YFP cell culture. Grids were put on a commercially available grid holder for convenient handling. Next, grids were sterilized by UV irradiation for 30 min and immersed in 1 mg/ml poly-L-lysine/0.1 M borate buffer (pH 8.5) in the dark overnight. After the poly-L-lysine coating, grids were washed with autoclaved distilled water three times, and kept in Hank's balanced salt solution (HBSS) until they were used for cell culture.

PtK2 (*Potorous tridactylus* kidney) cells expressing β-actin–Phi Yellow fluorescent protein (YFP) were purchased (Marinpharm GmbH). PtK2 cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum (FBS) (heat-inactivated at 56 °C for 30 min), 0.1 mM non-essential amino acids (NEAA), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Gibco) at 37 °C in a 95% air/5% CO_2 incubator. Cells (~2.4×10^6) were seeded onto poly-L-lysine-coated gold finder TEM grids in 100 mm-diameter cell culture dishes, and then cultured for ~32 h. In order to make cells thinner, culture medium was exchanged with serum free medium ~15 h before plunge-freezing of the specimen.

Different sizes of yellow-green fluorescent latex beads (0.1 μm-,1 μm- and 2 μm-diameter) were purchased from Invitrogen (FluoSphere Ex505/Em515).

TEM grids were plunge-frozen in liquid ethane at a temperature close to the liquid-nitrogen temperature using a Vitrobot™ (FEI). The blotting chamber conditions were set to 37 °C, 90% humidity, and a relatively long blot time of 30 s. After plunging, excess ethane was blotted from the grid using a small piece of filter paper in the cold nitrogen-gas atmosphere just above the surface of the liquid nitrogen surrounding the ethane cup. Grids were stored in liquid nitrogen until observation.

2.2. Transmission electron microscope

A standard TEM JEM-2010 (JEOL) equipped with a 200 kV LaB_6 electron gun was used for the basal unit of the hybrid microscope. The pole piece and objective lens column of the TEM were designed to allow insertion of the fluorescence microscope module into the TEM column. This associated with worsened optical constants Cs and Cc compared to those typically employed in conventional TEM (refer to Table 1). TEM experiments were conducted at an acceleration voltage of 200 kV. Images were acquired using a monochromatic CCD camera (FastScan F114;1024 × 1024, 14 μm/pixel; TVIPS). All cryo-TEM images were obtained at liquid-nitrogen temperature using cryo-transfer holder (Model 914, Gatan). Cryo-TEM images were taken with 5 μm defocus with an electron dose of 60 electrons/nm^2. Under the large defocus condition, the effect of optical constants Cs and Cc for the contrast transfer can be negligible and the contrast and resolution depends only on the defocus value and the electron source. The optical performance of the hybrid microscope at the 5 μm defocus therefore was quite similar to that of conventional 200 kV TEM equipped with a LaB_6 electron gun. The performance of the TEM unit is to be described in the Section 3.2 with characterization for resolution and contrast (refer to Fig. 2).

2.3. Fluorescence microscope

Our fluorescence microscope module, built in-house, was equipped with an objective lens (NA: 0.42, Mag: ×50; Mitsutoyo), a dichroic mirror (Olympus), optical filters (Olympus), a projection lens (Mitsutoyo), and a CCD camera (658 × 496 pixels, 10 μm/pixel; Andor); for additional details, see Results. For FL imaging, we employed a specific set of filters: excitation range, 460–490 nm; detection range, >510 nm. As a key feature of our hybrid microscope, the same specimen support (grid) as used for TEM experiments was employed.

2.4. Cathodoluminescence microscopy with TEM

The cathodoluminescence microscopy that can visualize samples in a TEM mode through a luminescence induced by electron irradiation, namely cathodoluminescence (CL), was achieved under a proper choice of modules for irradiation and detection: electron irradiation to and light detection from a single specimen. This type of imaging is, as mentioned previously, is novel and unique.

2.5. Cathodoluminescence spectroscopy and microscopy with STEM

For the comparison of TEM-CL and SEM-CL (or STEM-CL) imaging, a commercial STEM system equipped with a CL module (JEM-2000FX, JEOL) was utilized at an acceleration voltage of 80 kV. The electron beam has a diameter of ~10 nm with a beam current of 0.27 nA. Luminescence emitted from specimens was collected by a parabolic mirror and collected on a detector outside of the STEM. Emission spectra were measured by a CCD detector (1024 × 512 pixels, 15 μm/pixel).

3. Results

3.1. TEM pole-piece design compromising the TEM and FLM objective lenses

A FLM module with a lateral extension was inserted into the pole-piece gap of the objective lens of TEM (Fig. 1a). The TEM

Table 1
Parameters of two objective lenses.

EM objective (JEOL)	LM objective (Mitsutoyo Plan ApoSL)
Focal length: 10.9 mm	Magnification: ×50
Point resolution: 0.42 nm	Working distance : 10.5 mm, NA: 0.42
Cs: 10.5 mm	Focal length: 4 mm
Cc: 8.4 mm	Point resolution: 0.7 μm