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Light-microscope specimen holder with 3-axis rotation and small-angle control

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HIGHLIGHTS

- Specimen holders were developed for light-microscope observation.
- They allow multiple-axis rotation with small-angle control.
- Specimen tilting and rotation can be corrected while viewing.
- They are easily manufactured and installed on a conventional microscope stage.

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ABSTRACT

Background: Although recent developments in methodologies for light microscopy have enabled imaging of fine biological structures, such imaging is often accompanied by two types of problems. One is a tilting of the specimen with respect to the *x*–*y* plane (i.e. rotation around the *x*- or *y*-axis) such that the sample is not perpendicular to the optical *z*-axis, and the other is rotation around the *z*-axis that precludes optimal orientations for imaging and experimentation. These rotation problems can cause optical aberrations and hamper imaging experiments, even when the angular difference from the ideal position is small.
New method: In order to correct for these practical issues, we have developed a specimen holder with 3-axis (*x*–*y*–*z*) rotation for an inverted light microscope. This allows for full-range rotations of 2–4° for *x*-, *y*-axes, ~24° for *z*-axis, and a small-angle control of <0.1° for either axis.
Results: Using this device, we observed the cultured hippocampal neurons stained by immunofluorescence for a dendritic marker, or the sub-resolution fluorescent beads plated on a glass coverslip. The rotations and associated problems could be manipulated, while viewing the specimens by laser-scanning confocal microscopy.
Comparison with existing methods: This tilting/rotation device is easily manufactured and installed on a conventional microscope stage without requiring changes to the existing optical components. Similar devices with full capability have not been available.
Conclusions: It will be useful for imaging experiments with biomedical applications.

1. Introduction

Recent developments in methodologies for light microscopy have made high-resolution techniques, such as confocal and two-photon microscopy, accessible to most imaging scientists, and have enabled the imaging of fine biological structures. Optimizing the acquisition of such images often requires fine control over rotation of the specimen with respect to the three spatial axes. Such control is necessary for two reasons.

First, light microscopes typically require the plane of the specimen (*x*- and *y*-axes) to be perpendicular to the light path through an objective lens (*z*-axis). Non-perpendicular, tilted positioning interferes with the spatial range of the image field, and thus with the data acquisition. A perpendicular orientation of the specimen is especially critical in certain microscopy methods, such as: confocal fluorescence microscopy, where a single optical section has a

Abbreviations: MAP2, microtubule-associated protein 2; NA, numerical aperture; PBS, phosphate-buffered saline.

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thickness of less than 1–2 μm; total internal reflection fluorescence microscopy, where imaging is limited to a distance of less than 100 nm from the surface of glass coverslip; and reflection interference contrast microscopy, where imaging is limited to the interface between the coverslip and cell (Zenisek et al., 2002). Furthermore, tilted specimens create optical problems, such as comatic and astigmatic aberrations (Goodwin, 2007). Tilting-associated aberrations are more severe when cells in aqueous environment are imaged with a water-immersion versus oil-immersion objective lens (Arimoto and Murray, 2004). Thus it is especially important that the specimen plane is perpendicular to the light path in the cases of live or chemically fixed cells that are imaged in aqueous medium. Unfortunately, tilting problems are frequently encountered in practice (Arimoto and Murray, 2004; Brakenhoff et al., 2005; van den Doel et al., 1998), caused by inaccurate control in mounting specimens in an imaging chamber, inaccurate installation of the imaging chamber on the microscope stage, or flaws in manufacture of the imaging chamber and/or microscope stage.

Second, certain imaging experiments require rotation of the specimen around the *z*-axis, so that a specific orientation can be made. For example, live neurons can be imaged by confocal microscopy with the positions of long, apical dendrites adjusted such that they can be imaged with a pre-fixed position of line scanning. Also, such positioning might be important for imaging the effects of applying pharmacological reagents in a specified direction, such as from the dendrite to the soma, when the setup for applying extracellular solution is mechanically fixed to the microscope stage.

Partial solutions to these rotation problems around the *x*-, *y*- and *z*-axes have been adequate for meeting some individualized requirements. However, a full solution for small-angle control of the rotation around all 3 axes has not been easily available. In addition, an ideal device would be easily installed without changing existing optical components, and be applicable to conventional inverted microscopes, which have limited space, especially between the objective lens, specimen, and transmitted light components (e.g. a condenser). First, in existing devices that control rotation around one (Stagno and Friedenbach, 1979) or more axes, the specimen is located within a limited space (1 mm × 5 mm) (Skaer and Whytock, 1975), the specimen is attached to the outside wall of a glass capillary or fiber (Shaw et al., 1989; Staier et al., 2011), or rotation is applied to optical components other than the objective lens or specimen (Arimoto and Murray, 1996). These methods are either limited to few axes of rotations, or not readily applicable to most biological imaging experiments. Second, existing devices can control rotation of a field of view, by rotating the coupler for a camera. This can change the apparent *z*-rotation position with respect to the viewer (Duerstock et al., 2010) but does not change the rotation angle of the specimen with respect to the microscope stage. The *x*- or *y*-rotation cannot be controlled either. Third, some commercially available devices allow rotation around certain axes. These include rotating stages with one-axis (*z*-axis) rotation, and devices with three-axis rotations, for example, the Hexapod (Physik Instrumente) (Scheibe et al., 2011), the Multi-Axis Tilt Platforms (Newport), and the 6-Axis Nanomax Nanopositioner (Thorlabs). Universal stages with three-axis control were once available as well (Berbel et al., 1981; Kile, 2009). However, these devices were not designed for use on the stages of inverted microscopes, and the objective lenses could not approach the under-side of the specimen. Lastly, there are commercially available microscopes that allow limited rotation. For example, the Movable Objective Microscope (Sutter Instrument) allows an objective lens to rotate around the optical axis, and the Orbital Nosepiece (Prairie Technologies) allows an objective lens to be tilted. However, these products do not enable either 3-axis rotation or small-angle control, and they replace key optical components of the microscopes. Thus there has been a paucity of practically applicable specimen holders for general purposes.

We have built a simple device that makes it possible to correct for and readjust the specimen rotation on all three axes (*x*–*y*–*z*). This specimen holder is designed for an inverted microscope, and provides the ability to control rotation on the order of 0.02° around *x*- and *y*-axes, and the ability to control rotation on the order of 0.08° around the *z*-axis. To illustrate the usefulness of this device, we cultured mouse hippocampal neurons on a glass coverslip, immuno-fluorescently labeled them for the dendritic marker, microtubule-associated protein 2 (MAP2), and imaged the dendrites on a confocal microscope with rotation around three axes. In addition, we have prepared a device that provides the ability to rotate specimens around two axes (*x* and *y*), for an upright microscope, by modifying a commercially available product. We illustrate the usefulness of the *x*–*y* specimen-holder by imaging cultured glial cells. These two devices can be easily manufactured by a standard machine shop, will be inexpensive, and will be useful for not only neuroscience, but also cell biological and biomedical applications.

2. Materials and methods

2.1. Specimen holders

A specimen holder for three-axis (*x*–*y*–*z*) rotation was mounted in the rectangular opening (11 cm × 16 cm) of an inverted microscope stage (Axiovert 100 M, LSM 510 confocal system; Carl Zeiss MicroImaging GmbH), replacing the mounting frame. It was designed to accommodate an imaging chamber (e.g. RC-26; Warner Instruments). It was custom-built using (1) 2024 T4 aluminum for the main components, (2) phosphor bronze for the hinges between the layers ("a" and "c" in Figs. 1 and S2), and for the clips that fix the imaging chamber ("f" in Fig. 1), (3) brass for a cubic nut with a groove in the *z*-rotation mechanism (a part of "e" in Figs. 1 and S3C and D), and (4) stainless steel screws.

A specimen holder for two-axis (*x*–*y*) rotation was designed for imaging a thin specimen, such as that on a slide glass, and to be mounted on an upright microscope stage (Eclipse E800; Nikon). It was prepared by modifying a commercially available product used for optical alignment (NT55-459, tilt platform; Edmund Optics).

The angular changes in these holders were measured on a surface plate with a vernier protractor attached to a height gauge. The

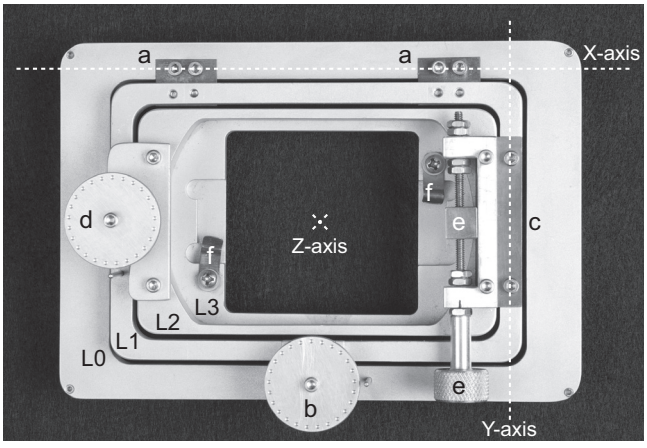

Fig. 1. Structure of the *x*–*y*–*z* specimen holder. This consists of four layers (L0: base layer, and L1–3). The labels indicate: (a) hinges for rotating L1 + L2 + L3 around the *x*-axis, (b) knob for rotating L1 + L2 + L3 around the *x*-axis, (c) hinges for rotating L2 + L3 around the *y*-axis, (d) knob for rotating L2 + L3 around the *y*-axis, (e) knob and cubic nut for rotating L3 around the *z*-axis, and (f) clips for fixing the imaging chamber. Dotted lines indicate approximate positions of the axes.