Intermediate Magnification Imaging System for Whole Organs/Organisms.

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There are many techniques for 3D imaging of biological specimens such as confocal, two-photon and, wide-field fluorescence microscopy, CAT scan, MRI, and optical coherence tomography. There are also many derivatives of these techniques, each having its strengths and weaknesses. Due to the differences in resolution, depth-of-field, and field-of-view, it is often difficult to compare images from the relatively high-resolution microscopy methods to the latter lower-resolution high-volume imaging methods. Effectively making this comparison could be very powerful in relating organ or organism level information to cellular level processes. Several microscope systems are being developed that bridge this gap. The system reported here has several advantages for 3-D imaging of large objects such as whole organs, organisms or embryos utilizing reflection, transmission and fluorescent imaging modes. It has higher resolution than NMR, and is relatively inexpensive. Additionally, the ability to use commercially available antibody/fluorescent markers including green fluorescent proteins, allows a large number of molecules to be labeled and their distributions imaged. The juxtaposition of proteins within objects, such as a mouse embryo, can be easily determined by using multiple labels and/or multiple imaging modes. With image algorithms, some borrowed from CAT and MRI imaging, as well as digital microscopic imaging, this technology has the potential to evolve and improve in terms of resolution, detection of a number of labels at lower levels, and the variety of specimen types and sizes that can be imaged. Instruments of this type will be especially important in bridging the gap between medical imaging and microscopy.

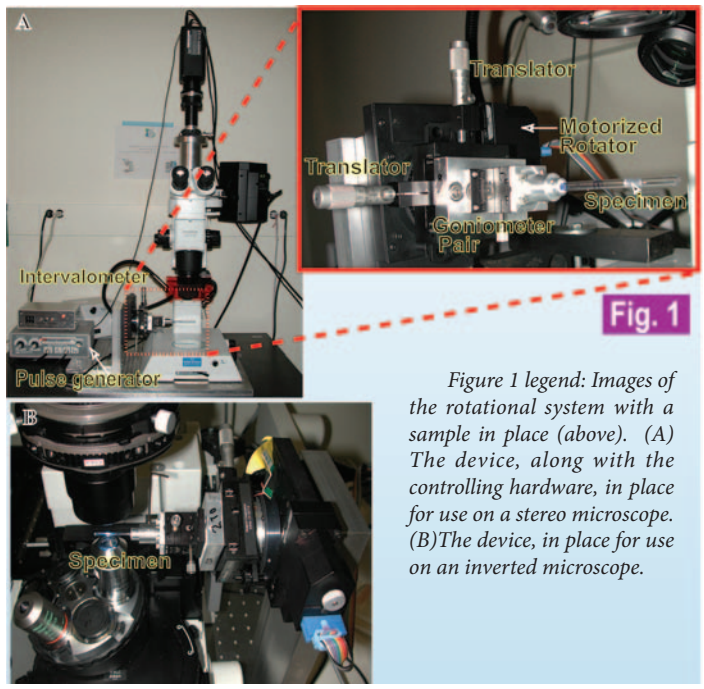

Figure 1 legend: Images of the rotational system with a sample in place (above). (A) The device, along with the controlling hardware, in place for use on a stereo microscope. (B)The device, in place for use on an inverted microscope.

Methods and material

Microscopy:

The general microscopic layout (Fig. 1) is similar to [5]. Basically, the specimen is held in a glass tube that is rotated 360° in the object plane. The specimen tube is held in a custom adaptor that is mounted to a two-axis goniometer for angular alignment. The goniometer is mounted perpendicular to the plane of motion of a two-axis translation stage providing y,z positioning. This combined mechanism is mounted on a precision rotator. The specimen tube is aligned to the microscope axis using the goniometer and translator, and the images collected as a function of rotation about the tube's long axis. An image sequence is collected conforming to the geometry of classic tomographic imaging. A TTL pulse is used to control the rotation of the specimen (model 13038 Oriel Corp., Stratford, CT) via a custom made intervalometer (Schematic 1) and a commercial pulse generator (model 4001 Global Specialties, Cheshire, CT). The degrees of rotation of the stage are controlled by the number of TTL pulses that it receives. The camera generates one pulse per image and therefore a method of amplifying and controlling the number of pulses per image was required. The single pulse from the camera is fed into the intervalometer, which then keeps that signal high for the desired time. The signal is then fed into the pulse generator, which continuously outputs TTL pulses till the signal goes low, *i.e.*, the timer from the intervalometer ends. In this method the amount of time on the intervalometer controls the

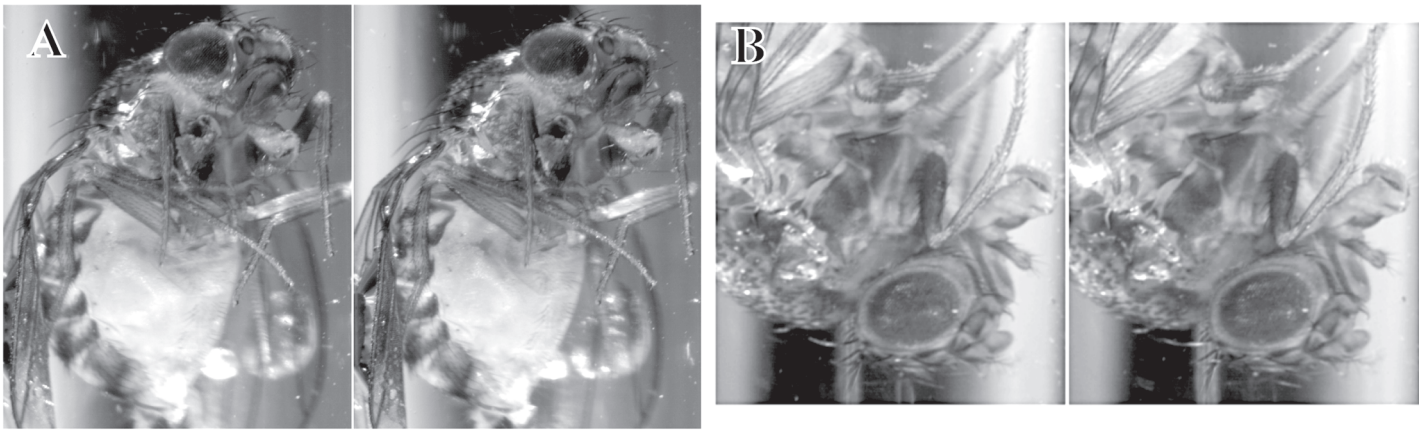

Figure legend 2: A common fruit fly (Diptera Tephritidae) was imaged in reflection mode on a stereoscope with images captured at 1.8° intervals to demonstrate the practicality of the system. A&B are two sets (low 10X & high 18X magnification) of stereo pairs demonstrating the ability to form true 3D images that accurately show the depth information in the specimen, vs. a conventional none stereo pair image.

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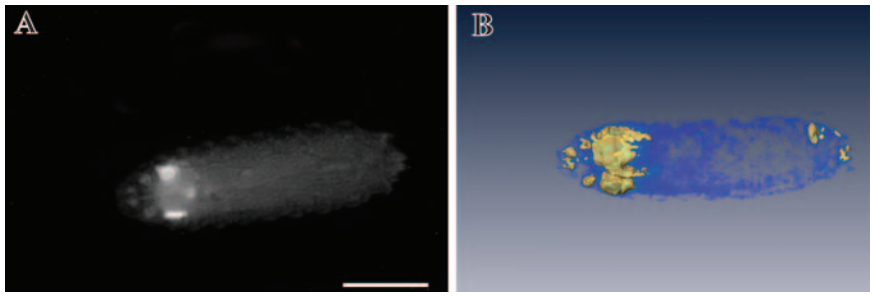


Figure legend 3: Single image, from a 1° per image rotational series of GFP expression taken on a stereo scope (A) in a larva and the resulting reconstruction (B). The GFP is localized to the brain and salivary gland. One of the challenges is to match the depth of field of the imaging system to the specimen to minimize aberration in the back projections. Another challenge is to excite the sample with enough light to penetrate the sample completely while minimizing photo bleaching. In this example the reconstruction is only from the outer 1/2 of the larva. Bar = 1mm

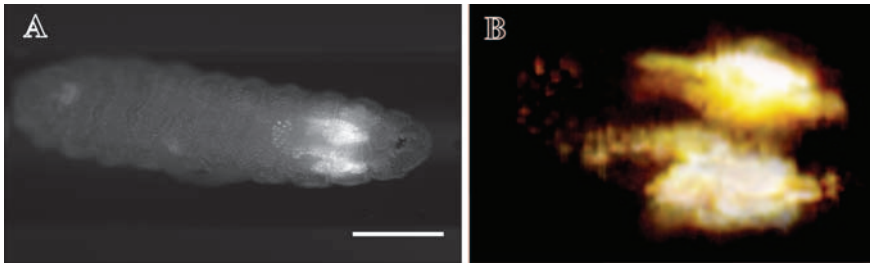


Figure 4 legend: Single image, from a 1° per image rotational series of GFP expression taken on an inverted scope (A) in a larva and the resulting reconstruction (B). The GFP is localized to the brain and salivary gland. The main difference between these images and the images in Fig.3 is that the specimen in this figure was completely sampled. The fluorescence imaging mode used with the inverted microscope is superior to the stereo scope in that the objective lens functions as the condenser as well. Bar = 1mm

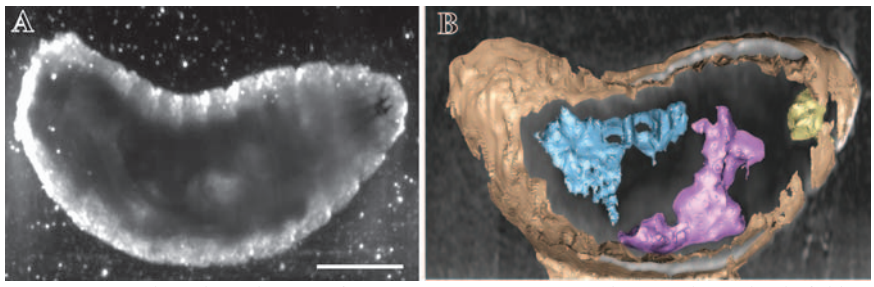


Figure 5 legend: Single image, from a 1.5° per image rotational series taken in bright field mode of a larva imaged with an inverted microscope (A) and the resulting reconstruction represented in surface rendering (B). The different colors represent different parts of the digestive system. The gold represents the mouth while the purple represents the stomach and the blue represents the lower position of the digestive track. Bar = 1mm

amount of angular motion of the rotational stage. To demonstrate the versatility of the system two different microscopes were used.

- A variable zoom dissecting microscope Fig.1A (SZX12 Olympus America, Center Valley, PA). The scope is equipped with fluorescent capabilities. The magnification was set to a level such that the entire specimen was within the depth-of-field. The N.A. of the front lens is 0.1. The images are collected with a 3 shot color camera (Spot 11.3, Diagnostic Imaging, Sterling Heights, MI). The software (Spot software Ver. 4.6, Diagnostic Imaging), which controls the camera, also generates a TTL pulse that is used to control the rotation of the specimen.

- An inverted compound scope Fig.1B (Nikon TE2000, Melville, NY). This scope is equipped with epi-fluorescence capabilities. The stage was removed to allow easier access. A 2X objective lens was used for all imaging (N.A. 0.1). The images are collected with a Roper HQ CCD camera (Roper Scientific, Trenton, NJ), which was controlled by ImagePro software (Media Cybernetics, Silver Spring, MD). The TTL pulse required to control the stage rotation was generated from a Uniblitz shutter (Vincent Associates, Rochester, NY) under the control of the ImagePro software.

Generation of tomograms from optical projections

Adaptation of the methods used for electron tomography to optical tomography proved to be non-trivial. The standard back projection algorithms work if the complete sample is entirely within the depth of field. Otherwise the algorithm needs to be modified to account for limited depth of field.

Alignment of the tilt series, however, was not straight forward. Fiducial alignment was initially attempted by the aid of reflective or fluorescent beads in the media. However the location of the fiducial markers in each projection was not well behaved, and lead to errors in alignment. This error was in the form of a systematic drift in the direction perpendicular to the optical and tilt axes. Alignment, then, had to be done manually by aligning to a single bead and adjusting the in-plane rotation of the projections so that the tilt axis was aligned correctly.

We theorize that the fiducial alignment failed due to refraction at the curved surface of the capillary wall containing the sample. This caused the apparent locations of the fiduacial markers to change systematically as the markers rotated to a position further from the tilt axis where the refraction error was greatest. This effect will also cause distortions in the reconstruction if the sample is of the order of the diameter of the capillary. For this reason samples were first embedded into small cylinders of agarose and then inserted in to a larger diameter capillary tube for the data collection.

The reconstruction was done by a single axis weighted back projection as described in [4]. Alignment and reconstruction were done using the SPIDER software package [2]. Visualization was done using the software package AMIRA (Mercury Computer Systems).

Fly strains

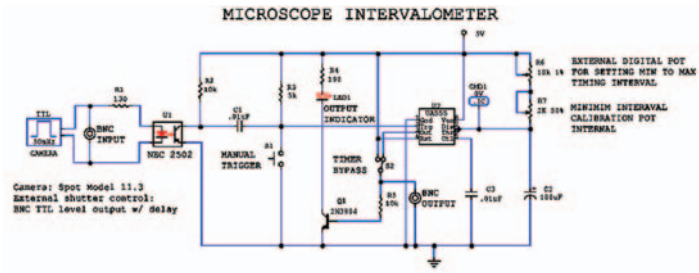
All work described in this study was performed on 3rd instar larvae ubiquitously expressing EGFP-tagged histones as previously described [9] . Flies were maintained under standard conditions. The larvae were mounted in 4% agar inside the capillary tubes.

Conclusions

Optical Projection Tomography (OPT) is a method for forming 3D images and renderings by combining optical microscopy with tomographic reconstruction methods.

Advantages are:

- Ability to reconstruct large objects, here larva (3mm x 1mm) in both fluorescence and transmitted light modes, with resolution of ~ 125μm. These reconstructions would be extremely difficult or impossible with other methods such as 2 photon confocal microscopy.



Schematic 1 legend: Schematic of the microscope intervalometer's interval timer, based on the industry standard NE555. Optical isolation and AC coupling were necessary to condition the camera's output pulse and prevent false triggering of the timer IC. The interval for timing pulses (i.e. the stage movement pulses) is determined by the setting of R6, an external 10K digital potentiometer. Adjustment of R6 varies the interval between 100 ms and 1000 ms. Manual trigger, output on indicator and an always on bypass were designed in for added flexibility.

- Relatively, low cost (<$10 K for an entire system, assuming existing microscope and CCD camera).
- Portability, the system can be easily moved between different type of microscopes depending on the image modality needed and resolution requirements.
- Allows large objects such as whole embryos or lava (as compared to either confocal or wide field) to be imaged.
- Higher resolution when compared to NMR or CAT scans.
- Allows the specimen to be imaged both with transmitted and Epi-illumination.

With image algorithms, some borrowed from CAT and MRI imaging, this technology has a lot of room to evolve and improve both in terms of detection ability and specimen types and sizes. While initially the algorithms are rather simple and easily available, as our needs expand we will seek to developed methods that allow higher magnification, such that only half the sample will be contained within the depth-of-field.

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