Fluorescence microtomography: Multi-angle image acquisition and 3D digital reconstruction

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ABSTRACT

We have developed a prototype fluorescence microscope which, using tomographic image acquisition and reconstruction techniques, can automatically combine conventional and/or confocal image stacks taken at a number of orientations into a single, very-high-resolution 3D image. We use the term "microtomography" in a broad sense to denote digital image reconstruction from multiple imaging operations which are not necessarily projections. Our system holds a biological specimen inside a thin capillary tube which is rotatable over a 360 degree range beneath an immersion objective. 3D fluorescence image data volumes are acquired by frame-grabbing a through-focus series of 2D images at each angle of rotation. Digital reconstruction of the multi-angle data volumes produces a single very-high-resolution 3D image and involves algorithms which perform rotation, interpolation, alignment and normalization operations in frequency (Fourier) space.

Keywords: 3D microscopy, tomography, fluorescence microscopy, 3D digital image reconstruction, 3D Fourier transforms

1. INTRODUCTION

The relatively poor axial resolution of conventional optical microscopes is well known and largely arises from the limited angle of illumination and detection imposed by the numerical aperture (NA) of microscope objectives. Efforts to surpass this limitation have led, in recent years, to the development of new techniques such as confocal microscopy and digital image deconvolution, both of which provide improvements but still do not yield axial resolution (i.e. depth resolution) equivalent to that obtained in the lateral direction. Another approach, described in this paper, is to acquire a series of images of a specimen over multiple angles of view, then use digital image reconstruction techniques to combine the multiple views into one very-high-resolution image. Because of its similarity to x-ray tomographic techniques used in medical diagnostics, we have called this system of optical image acquisition and digital reconstruction "microtomography."

1.1. Why fluorescence microtomography improves axial resolution

The improvement in axial resolution can perhaps best be explained diagrammatically (Fig. 1). For a conventional fluorescence microscope, the 3D point spread function (PSF) represents the extent by which the image of a single fluorescent point-like object will be broadened laterally and elongated axially. The extensive elongation of the PSF in the axial direction indicates that the resolution in depth is much poorer than the lateral resolution. Taking the Fourier transform (FT) of the PSF yields the 3D optical transfer function (OTF) of the system which in this case is "doughnut" or "bagel-shaped". This flattened structure represents the range of 3D object spatial frequencies which can be "transferred" by the optical system and reproduced in the image, with the lowest (zero) order frequencies located in the center and the higher frequencies (both positive and negative) radiating outward along axes corresponding to the three spatial dimensions. In particular, the flat rather than spherical shape of the 3D OTF indicates that there are certain object spatial frequencies which will be lost by the optical system, in this case those which correspond to features that vary in depth (i.e. close to the axial direction). These frequencies are often termed "the missing cone" and features in the original object having these frequencies will not be represented in the image. Figure 1(b) demonstrates how fluorescence microtomography alleviates this problem by combining multiple views of the specimen from a range of angles, resulting in an OTF which is a combination of overlapping "bagels" thus filling in the missing cone of spatial frequencies and providing axial resolution which is as good as the lateral resolution of the system.

1.2. Aims of our approach

Previous research by Shaw *et al.*,[1] into reconstruction of conventional (i.e. non-confocal) fluorescence microscope images, uses a specimen mounted on the outside of a precisely-aligned narrow rotatable tube. This allows only a limited range of tilted views (± 45 degrees) but does obtain a stack of image slices at each angle of tilt and then uses sophisticated algorithms for rotation, alignment and normalization of the datasets. Reports by Bradl *et al.*[2,3] rotate the specimen over 360 degrees inside a capillary tube but are limited in that they use a relatively imprecise algorithm (i.e. thresholding) for finding the center of rotation. A third approach

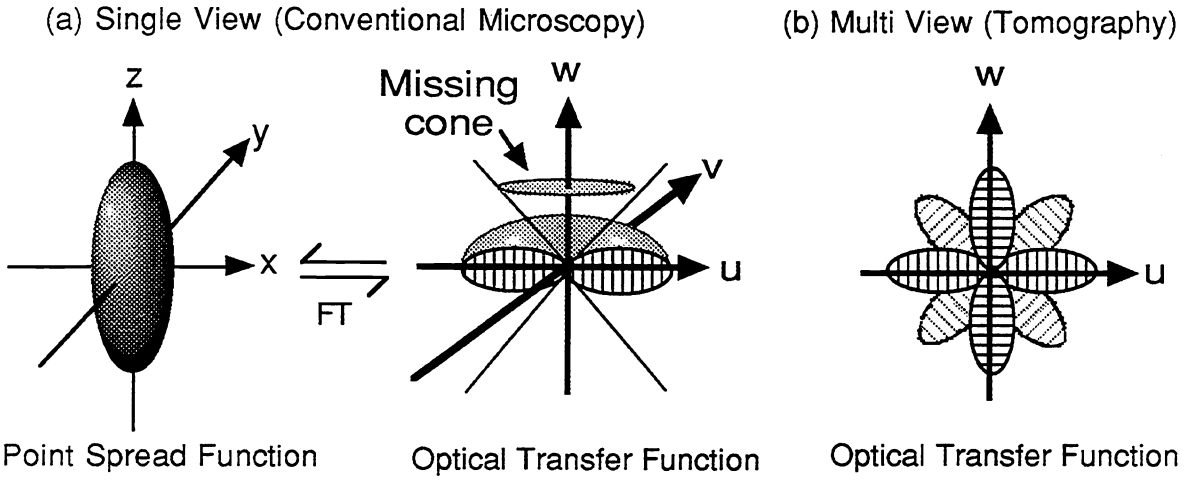

Fig. 1. (a) The point spread function (PSF) of a conventional fluorescence microscope and its Fourier transform, the optical transfer function (OTF). The reduced axial resolution of the system is represented by the vertically elongated PSF (i.e. along the z-axis) and by the corresponding missing cone of spatial frequencies in the OTF. (b) The OTF of a multi-angle view (tomographic) fluorescence microscope has no missing cone and thus has equivalent resolution in all directions.

by Kawata[4] is limited by using a microscope specimen slide and a small range of illumination angles, but is interesting in that it begins to explore tomographic imaging in transmission using oblique illumination to recover phase information.

Our principal aims for this project were the following:

(1) To design a simple mechanical attachment to a commercial microscope that would rotate the specimen over a full 360 degrees and that would not require expensive aligning optics but would instead utilize our comprehensive, automated, digital alignment and reconstruction algorithms,
(2) To extend fluorescence microtomography into the confocal realm to give further resolution improvement in the lateral as well as axial direction,
(3) To generalize microtomography so that non-fluorescent (phase) objects can be imaged using visible light.

2. SPECIMEN HOLDER AND OPTICAL SETUP FOR MICROTOMOGRAPHIC IMAGING

By placing the specimen inside a carefully drawn capillary, viewing from any angle within the 360 degree range is possible. The capillary used is a 1.5mm borosilicate tube which has been precisely drawn down to a diameter of about 90 microns which allows a short working distance objective to view from all angles. The rotation angle of the tube is accurately controlled (to within 0.5 degrees) by a manually operated gear. The simplicity of the modified specimen holder avoids the costs of precision opto-mechanical alignment and is compact enough to be easily attached to most commercial microscopes. Using this approach we have drastically reduced the mechanical complexity and concentrated on software compensation for mechanical defects wherever possible.

A critical factor in the design of the microtomography system is that there must be uniform sampling of the specimen in all directions (x, y and z). This requires a linear focusing device and a distortion-free objective. In our system we use a calibrated piezoelectric transducer to focus a 40x, 0.9 NA glycerine-immersion objective (Zeiss Plan Neofluar) through 1.1μm intervals. For each viewing angle, 64 image slices are acquired using a CCD camera mounted on a commercial fluorescence microscope (Zeiss Axioplan).

The specimen used for this preliminary study was a cluster of five fluorescein-labelled beads, each 10μm in diameter. The beads were mounted in a glycerine-agarose mix to prevent movement of the specimen during rotation as well as to match refractive indices between the borosilicate capillary and the glycerine immersion medium (refractive index = 1.47) to reduce aberrations. Image stacks were acquired at four different viewing angles spaced 45 degrees apart and were digitized and processed using a high-speed graphics workstation (Silicon Graphics Crimson). The processing and reconstruction algorithms were written and incorporated into a commercial 3D imaging package (Vital Image's VoxelView).

3. DIGITAL IMAGE PROCESSING AND RECONSTRUCTION OF MULTI-ANGLE IMAGE DATA VOLUMES

We have developed a comprehensive algorithm that combines the data stacks acquired from each angle of view into a final high-resolution 3D image. The algorithm performs several operations which are summarized in the following outline and described in detail in the subsections below:

(1) Rotation and interpolation of each stack of images to coincide with the data volume from the original viewing angle,
(2) Precision alignment and registration (in three dimensions) of the rotated data volumes,
(3) Normalization, noise removal and summation of the 3D volumes,
(4) Visualization of the final combined 3D image.

3.1. Rotation and interpolation of 3D data volumes

In tomography multiple datasets of one object are taken at different angles. Since these data must be combined in one fixed array, all of the subsequent datasets must be rotated back to align in register with that of the original viewing angle. In optical micro-tomography, the information from each Fourier transformed dataset (in the frequency domain) corresponds to a single OTF "bagel" with the optical (viewing) axis passing through the central hole. In our system the OTFs from all the viewing angles are initially aligned in the same direction as the OTF of figure 1(a), due to our procedure of rotating the specimen capillary tube rather than moving the microscope objective lens about the specimen. Thus the desired outcome of the digital rotation and interpolation step is to obtain the star pattern of 3D bagel-shaped OTFs shown in cross-section in figure 1(b). Specifically, this entails rotating each data stack and resampling onto the same grid as that used in the initial view volume.

Existing methods for accomplishing this task include bilinear and bicubic interpolation. Although these sometimes are combined with an x-y-x shear method to make data handling more efficient, they fall short of ideal because of their tendency to lose high frequency (i.e. high resolution) information. To alleviate this problem we have developed a pure Fourier interpolation algorithm which can be summarized in the following steps in frequency (Fourier) space:

(1) The 3D data volume is prepared by a special (non-zero) padding scheme,
(2) The rotation and Fourier interpolation is implemented by a three pass (x-y-x) shearing algorithm which allows a special feature of the Fourier shift theorem to be applied to the corresponding one-dimensional rows and columns of the 2D image transforms from each data stack. More specifically, this entails first reslicing vertically through a stack of (horizontal) 2D images from one view volume (Fig. 2) to form a new stack of 2D (now vertical) slices,
(3) The first of these new 2D slices is further broken down into a stack of 1D horizontal rows,
(4) The first skew operation (i.e. step one of the rotation, the x-skew) takes the fast Fourier transform (FFT) of the first of these rows and stores it in two arrays, one containing the magnitude and one containing the phase of the FFT. It then uses the Fourier shift theorem to calculate the appropriate "phase factor" corresponding to the amount of shift or "skew" being applied to this particular row. Each value in the row's FFT phase array is multiplied by this phase factor and then an inverse FFT is performed,
(5) Step (4) is repeated for all the rows in the first of the 2D slices, then steps (3) and (4) are repeated for all of the 2D vertical slices in the volume,
(6) The second skew operation (the y-skew) works on these x-skewed 2D slices in a similar way only now the image data are grouped into vertical columns instead of horizontal rows. These columns are Fourier transformed one at a time, shifted down and the appropriate phase factor calculated and applied prior to the inverse FFT, just as in the previous x-skew. This again is repeated for all columns in all of the 2D slices,
(7) Finally, a third skew operation, another x-skew, is performed and the resulting inverse FFTs give the properly rotated stack of image slices, which is then resampled onto the original data volume grid.

In addition, the algorithm addresses aliasing issues and edge effects which can cause artefacts in the resulting image stacks.

The advantages of our algorithm are primarily in the speed of computation obtained by performing FFTs on one-dimensional data (i.e. rows and columns) rather than 2D or 3D datasets. In addition, calculation of the phase factor for each skew operation is straightforward, as it increases linearly with the amount of shift. Finally, as mentioned previously, the algorithm preserves the high spatial frequencies and hence the fine detail of the original images.

3.2. Precise alignment of 3D data volumes

Because the specimen capillary tube does not rotate precisely about a single point on the optical axis, it is necessary, as a next step, to realign (i.e. shift) the rotated data volumes so that they are precisely in register in three dimensions. This step requires using some sort of correlation algorithm on pairs of data volumes, and in our system we compared two approaches: phase-correlation and cross-correlation. These techniques are well known in signal and image processing and have been recently described for

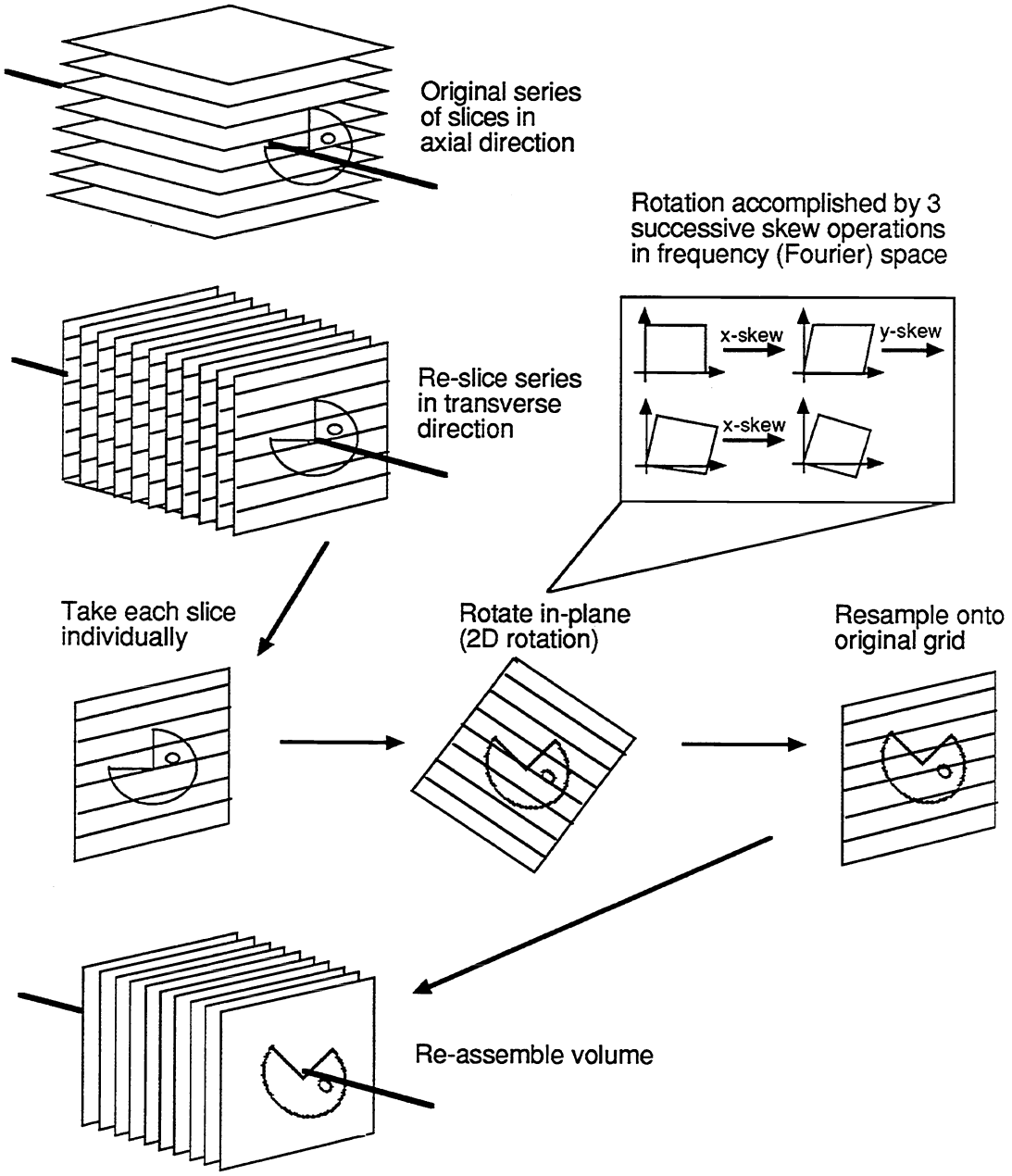

Fig. 2. Schematic diagram of our rotation and resampling algorithm which performs 3 successive skew operations by successively shifting the 1D FFTs of the rows and columns in each 2D slice.

optical tomographic applications by Shaw *et al.*[1] Both approaches require performing a 3D FFT on each of the data volumes to be correlated.

In order for correlation algorithms to work effectively, it is necessary to apply them only to regions where the two data volumes overlap. In the case of our multi-angle-view microtomographic system, this can best be illustrated by figure 1(b), in which some of the spatial frequencies in any one 3D OTF overlap with those from the next rotation angle. In order to extract the regions of overlap between data volumes from two successive angles of view, we have calculated a 3D mask in Fourier space which defines

the overlap regions between the two OTFs. This mask is given the value of 1 inside (i.e. where there is an overlap between the data volumes being aligned) and a value of 0 outside. Therefore, when the mask is multiplied by each of the Fourier transforms of the image volumes to be aligned, it extracts from each their common spatial frequencies (i.e. those angles of illumination and detection they had in common due to the large NA of the microscope objective used).

Because our experimental microtomography system uses a low-precision mechanical rotary specimen-holder, we found that there were fairly large shifts between the resulting re-rotated image volumes and hence the phase-correlation algorithm did not give a strong enough result to effect a precise re-alignment of the volumes. Normally, using phase-correlation is an advantage because it uses only the phase information of the Fourier transform and avoids the magnitude information (used by cross-correlation) which (for typical images) may cause the lower spatial frequencies to be much more heavily weighted than the high spatial frequencies, thereby reducing the accuracy of the alignment process. However, by using an additional technique for finding the center of the broad peak of a cross-correlation, we were able to get reasonable alignment information for our image volumes.

3.3. Normalization, noise removal and summation of 3D data volumes

The next step required in the 3D tomographic reconstruction process can also be conveniently described in Fourier space. Certain spatial frequencies are imaged by a physical microscope system while others are not, and the aim of microtomography is to fill the regions of missing spatial frequencies by producing several datasets taken at different orientations. The exact shape of the OTF is again determined by the imaging system but this time not only is the shape of importance (as for the mask used for alignment of datasets) but also the amount of amplification of each spatial frequency which is defined by the system's transfer function. These variations in amplitude in the OTF must be "normalized" before combining the data volumes in order to prevent the low and mid spatial frequencies (i.e. those with higher amplitudes in the OTF) from overpowering and effectively deleting the high spatial frequencies from the final combined image. The following is a general overview of the steps performed in this section of the reconstruction process:

(1) Calculate the approximate 3D incoherent transfer function for our high NA optical system based on Sheppard *et al.*[5],
(2) Take the 3D FFT of each rotated and aligned data volume,
(3) In frequency (Fourier) space, divide each volume's FT by the incoherent transfer function calculated in step (1). As part of this process, invoke a Gaussian filter to reduce noise which arises from the sharp boundaries of the transfer function. Also compensate (normalize) for the overlapping areas in Fourier space (between adjacent data volumes) and then sum the volumes.
(4) Inverse FFT to produce the final reconstructed image.

3.4. Visualization of final reconstructed volume

The final combined image volume was further enhanced and rendered for visualization using a standard 3D imaging package (VoxelView).

4. RESULTS AND DISCUSSION

The results of our initial experiments using a cluster of 10μm diameter fluorescent beads and four angles of view (rotated successively by 45 degrees) are illustrated in Fig. 3. The upper and lower pairs of images each show, at left, an original single-angle 3D view of the beads and, at right, the microtomographic, multi-angle reconstructed 3D volume, displayed at the same direction of view. The single-angle volume at top left corresponds to a view along the optical axis and, therefore, little distortion is seen although the beads are not well-separated. The equivalent multi-angle tomographic reconstruction (top right) has better delineation of the beads, however, some discontinuities (due to our algorithm's method of dealing with such things as edge effects) appear as breaks in what should be a smooth bead surface. Further experimenting and fine tuning of the noise reduction algorithms should improve this defect. The single-angle volume (bottom left) is displayed here as a side view and shows the beads elongated vertically, which corresponds to the reduced resolution of the microscope in the axial direction. The reconstructed tomographic volume (bottom right) eliminates most of this axial elongation artefact and shows a cluster of largely spherical beads.

5. CONCLUSION AND FUTURE PROSPECTS

We have shown it is possible to use a relatively low-precision, rotatable specimen tube mechanism in conjunction with a piezo focusing device on a conventional fluorescence microscope to produce a series of data stacks acquired from a range of viewing angles. A single very-high-resolution 3D image can be produced by combining these data volumes using an efficient digital reconstruction algorithm which performs rotation, interpolation, alignment and normalization operations in frequency (Fourier) space.

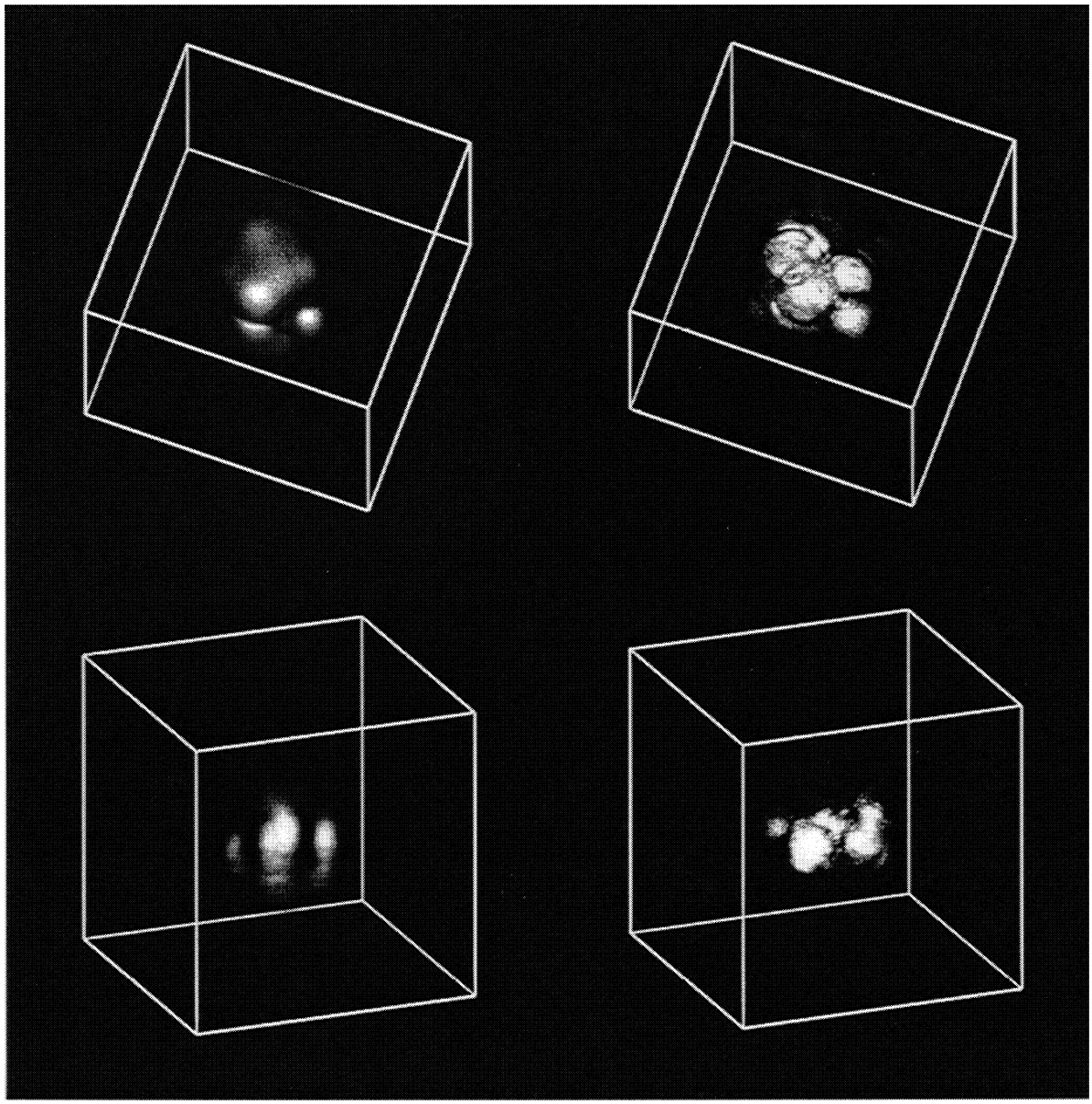

Fig. 3. Comparison of 3D single-angle view fluorescence microscope images of 10μm beads with 3D multi-angle micro-tomographic images of the identical specimen. (Top left) a single-angle volume viewed from the top (i.e. nearly parallel to the optical axis) shows little distortion but beads are not well-separated. (Top right) multi-angle tomographic reconstruction shows better delineation of beads but also has discontinuities due to the algorithm's inaccurate method of dealing with edge effects. (Bottom left) a side view of the single-angle 3D volume showing axial (vertical) elongation of beads due to missing cone of spatial frequencies in the OTF. (Bottom right) multi-angle tomographic reconstruction removes this artefact.

In the near future, this specimen, holder and focusing system will be mounted on a BioRad confocal microscope and the experiment repeated. It is expected that even further gains in axial resolution (and possibly lateral resolution) will be obtained due to the increase in spatial frequencies in the confocal OTF. We also intend to explore if a similar approach can be used for imaging non-fluorescent specimens. For example, the possibility of using microtomography to detect phase information from living cells is intriguing, but requires further development of theory to determine if phase retrieval is possible.

6. ACKNOWLEDGMENTS

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7. REFERENCES

1. P. J. Shaw, D. A. Agard, Y. Hiraoka and J. W. Sedat, "Tilted view reconstruction in optical microscopy," *Biophys. J.* **55**:101-110, 1989.
2. J. Bradl, M. Hausmann, V. Ehemann, D. Komitowski and C.Cremer, "A tilting device for three-dimensional microscopy: Application to in situ imaging of interphase cell nuclei," *J. Microsc.* **168**:47-57, 1992.
3. J. Bradl, M. Hausmann, B. Schneider, B.Rinke and C.Cremer, "A versatile 2π-tilting device for fluorescence microscopes," *J. Microsc.* 173: 211-221, 1994.
4. S. Kawata, "The optical computed tomography microscope," *Adv. Opt. & Electron Microsc.* **14**:213-248, 1994.
5. C.J.R. Sheppard, M. Gu, Y. Kawata and S. Kawata, "Three-dimensional transfer functions for high-aperture systems," *J. Opt. Soc. Am.* **11**: 593-598, 1994.