High-resolution real-time dual-view imaging with multiple point of view microscopy

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Abstract: Most methods to observe three-dimensional processes in living samples are based on imaging a single plane that is sequentially scanned through the sample. Sequential scanning is inherently slow, which can make it difficult to capture objects moving quickly in three dimensions. Here we present a novel method, multiple point-of-view microscopy (MPoVM), that allows simultaneous capturing of the front and side views of a sample with high resolution. MPoVM can be implemented in most fluorescence microscopes, offering new opportunities in the study of dynamic biological processes in three dimensions.

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1. Introduction

Many biological processes, from the molecular to the organism level, occur in three dimensions on time scales of milliseconds. Capturing and quantifying such processes requires fast and accurate three-dimensional imaging. Conventional fluorescence microscopy is in general the technique of choice to study live cells, but most current fluorescence microscopy methods, such as wide-field, light-sheet, confocal and spinning-disk fluorescence microscopy are inherently slow, since they depend on the consecutive imaging of multiple sample planes to reconstruct a volume [1]. Several refinements of these techniques, including dual-view light-sheet microscopy [2–4] and swept confoocal-aligned planar excitation microscopy [5] allow imaging of spatially separated parts of large volumes, which can reduce the time needed to image a full volume, but these techniques still require scanning. Novel approaches retrieving three-dimensional information without the need for scanning are necessary to push forward the limits of observation such that such fast biological processes can be tracked with
fluorescence microscopy. Light-field microscopy offered one of the first solutions to this challenge, although with limited spatial resolution [6]. Several techniques followed and were based on the simultaneous imaging of two or more planes in the sample. In a first implementation, several parallel planes are recorded simultaneously and combined to achieve volumetric imaging [7–9]. Currently this approach allows imaging of a volume of a few micrometers deep. The field of view is usually limited since all planes are imaged side by side on a single camera [9,10], and the spatial resolution obtained is at best the one of wide-field microscopy. In a second type of implementation, two or more oblique planes are recorded simultaneously [10–12]. Although in this implementation volumetric imaging is only obtained close to the intersection of imaging planes, the use of oblique planes provides axial information that can be used to improve axial resolution [12]. Moreover, since usually only two planes are imaged side by side on a single camera, a relatively large field of view can be obtained. Oblique imaging also allows the direct observation of the object along the axial axis of the microscope objective, which can be beneficial for some applications [13,14]; in contrast, obtaining the same view with conventional microscopy requires sequential imaging of the object along the axial axis, which is slow.

Oblique imaging in wide-field microscopy has been achieved in several ways. In a first set of methods, refocusing was adapted [15,16] to oblique imaging. In the original version of refocusing, the Wilson group proposed to use two facing objectives (the first objective to refocus the object and the second objective to image the refocused object, or to use a single objective facing a mirror to achieve the same goal [15]. For oblique imaging, the combination of two facing objective has been modified by tilting the second – imaging – objective with respect to the refocusing objective [17], whereas in the configuration using a microscope objective facing a mirror, the mirror is tilted [13,18]. One limitation of these methods is that the information contained in the pupil of the imaging objective is clipped when oblique planes are refocused [18]. As a result, the axial resolution cannot exceed that of wide-field microscopy. In another set of methods, oblique imaging is obtained by means of a micro-mirror positioned in the vicinity of the object [10–12,14,19]. The mirror is usually oriented at 45° with respect to the main axis of the objective to provide a tilted view of the object. As a result, refocusing the microscope on this mirror image of the object provides oblique imaging of the object. Here, the use of a mirror provides additional axial information that can improve axial resolution [12]. In configurations where only one objective is used in combination with a tilted mirror, only one imaging plane is available. As a result, simultaneous imaging of the object with conventional and oblique orientation can only be achieved when the object is extremely close to the mirror, which is only possible for very small objects such as bacteria [12]. This limitation was overcome by using two facing objectives in combination with a micro-mirror [14]. In this implementation, however, exact positioning of the object with respect to the micro-mirror is crucial [20], which presents challenges likely affecting spatial resolution.
2. MPoVM

Here we propose a different approach, Multiple Point-of-View Microscopy (MPoVM), that combines the use of a micro-mirror in the vicinity of the object with refocusing. The micro-mirror allows side-view imaging of the object [20], while refocusing allows visualization of direct-view and side-view images at the same time, using a single camera, Fig. 1(a).

2.1 MPoVM setup

We implemented MPoVM in a conventional, laser-illuminated epifluorescence microscope by positioning a micro-mirror consisting of multiple 120° grooves above the sample, Fig. 1(b). The main microscope objective, the one closest to the object, was positioned such that the tilted image of the object was in its focal plane, Fig. 1(a). Fluorescence emitted by the object was collected via the micro-mirror or directly, in both cases by the main objective. After the objective, the fluorescence signal was split into two light beams by a polarizing beam-splitter cube. The first beam, which had passed through the cube, pink path in Fig. 1(b), was reflected on a flat mirror after a quarter-wave plate and a second objective, the refocusing objective. The reflected beam went another time through the objective and the quarter-wave plate (resulting in a 90° rotation of the original polarization) and was reflected by the polarizing beam-splitter cube towards tube lens and camera. By adjusting the distance between mirror and refocusing objective, an in-focus image of the sample was created on one half of the camera, resulting in a front-view image of the object, Fig. 1(a). The second beam, reflected by the polarizing beam-splitter cube, passed through a quarter-wave plate and was reflected by a tilted mirror, cyan path in Fig. 1(b). After passing through the quarter-wave plate for a second time, the polarization had been rotated by 90°, allowing the beam to pass the beam-splitter cube. This second beam was imaged on the second half of the camera by the same tube lens as the first beam, resulting in an in-focus side-view image of the object. Since both views are imaged side by side on a single camera, the field of view of each view is half that of
conventional microscopy using the same camera. In our implementation, the angle between the front and side views was 60 degrees, but this can be modified by using another micro-mirror design or by tilting the mirror in front of the refocusing objective [13,18]. It is important to note that this setup is straightforward and can be readily incorporated in an existing fluorescence microscope, without the need for expensive or custom optics.

2.2 MPoVM spatial resolution

On basis of the optical design of MPoVM, combining two views of the sample, both with ellipsoid-shaped point-spread functions (PSFs), with long axes at a substantial angle, we expected an improvement of the spatial resolution, in particular in the lower-resolution axial direction. To test this aspect of our instrument, we measured the PSFs of front and side views using small (40 nm) green-fluorescent microspheres located on the coverslip and imbedded in water Fig. 2(a). The front view was obtained with refocusing, using the second objective. Although it had been demonstrated that refocusing works best with respect to resolution when matching objectives pupils [15], we found that a combination of 60X NA 1.20 water immersion main objective and a 40X NA 1.30 oil immersion refocusing objective, which fitted our optical layout better, resulted in a high-quality PSF over a refocusing range of several tens of micrometers, Figs. 2(c) and 2(d). The side view was obtained from the tilted-object plane via the micro-mirror. Imaging in this way using high numerical aperture objectives is known to result in slight geometrical aberrations [20], which are evident from the slightly asymmetric PSF measured for the side view, Fig. 2(a). These aberrations are negligible for objects close to the micro-mirror [12] (see Appendix). Intersecting the side-view PSF with that of the front view resulted in a two-fold improvement of the axial resolution compared to wide-field microscopy, while the lateral resolution was not affected, Fig. 2(b). The focal volume was $34 \pm 4\%$ smaller (defined as the volume of the PSF with intensity larger than half the maximum intensity). Close to the micro-mirror, the performance improves significantly and the combined PSF is 1.5 times larger in the axial than in the lateral direction, outperforming confocal microscopy axial resolution in these conditions (with sparsely distributed particles, using an objective with the same NA and the smallest pinhole configuration [1]; see Appendix).
Fig. 2. MPoVM views spatial resolution. (a) MPoVM point-spread functions (PSFs) obtained by imaging a 40 nm green-fluorescent microsphere. Left (pink frame): xz-projection of object-plane PSF (imaged using refocusing). Right (cyan frames): xz- and yz- projections of the tilted-object plane PSF. The separation between the two planes was 30 µm. Scale bars, 1 µm. (b) Projections (left: xz; right: yz) of the iso-intensity surfaces of the PSFs at half-maximum intensity. Blue: traditional wide-field fluorescence microscopy; pink: using refocusing; cyan: via the micro-mirror (no refocusing). Data obtained from 2D Gaussian fitting of image stacks; dots correspond to individual data points (measurement error smaller than the dots), curves are interpolations. (c-d) Effect of refocusing on spatial resolution. In this experiment, 40 nm green-fluorescent microspheres were fixed on a cover-glass surface and imbedded in water. The main objective was defocused from the coverslip surface at different distances (the refocusing height), and refocusing was applied to bring the microspheres back into focus on the camera plane. (c) Full width at half maximum of the images obtained from the microspheres at different refocusing heights; width measured in x- (red) and y-directions (black; errors bars are standard deviations). The dashed blue line corresponds to the PSF width using traditional epifluorescence imaging under the same conditions (line thickness corresponds to standard deviation). (d) Same as (c), for the full width at half maximum of the PSFs in the z-direction.

3. Application of MPoVM to biological samples

To demonstrate the potential of MPoVM, we imaged living C. elegans, a transparent, ~1 x 0.1 x 0.1 mm³ multicellular organism. We show that MPoVM can be advantageously used in several situations and allow observations that would be difficult to achieve with conventional microscopy.

3.1 Three-dimensional tracking of particles

In a first experiment, we tracked particles excreted by C. elegans diffusing in three dimensions. Three-dimensional tracking of particles is an important problem that has been tackled before using approaches involving astigmatism [21,22], simultaneous imaging of multiple, shifted parallel object planes [23], interferometry [24] or multiview by employing micro-mirrors [11]. In the multiview scheme [11] or using MPoVM the particles are localized by determining their center of mass in both (tilted) views, which is less stringent on signal-to-noise ratio than the more complex fitting algorithms used in the other methods [21–23]. MPoVM’s ability to track in three dimensions in real time is demonstrated in Fig. 3(a) and Visualization 1 where a diffusing EGFP-filled vesicle, excreted by C. elegans is imaged at five frames per second. The mean squared displacement could be calculated for all three dimensions from the position time trace. Note that the attainable frame rate is limited by
camera specifications and signal intensity, which makes tracking of very fast diffusing particles using MPoVM feasible.

![Fig. 3. MPoVM imaging of living C. elegans expressing EGFP-tagged OSM-3 kinesin motor proteins. Pink frames contain front views, cyan frames side views; orientation as indicated in the images. (a) 3D tracking of a particle excreted by C. elegans. Top: simultaneously recorded front-view and side-view images corresponding to the image sequence of Visualization 1. Scale bars, 2 µm. Bottom left: particle position extracted from image sequence, color coding follows time from red to dark blue (total duration 3.2 seconds; see Visualization 1; movie recorded at 5 frames per second; top: front view, bottom: side view; scale bar, 2 µm). Bottom right: mean squared displacement as a function of time in x (light grey), y (dark grey) and z (black) directions; error bars: standard deviation. (b) Velocity determination of kinesin motors driving intraflagellar transport. Top left: time-averaged images corresponding to image sequence in Visualization 2 (movie recorded at 5 frames per second and sped up 6 times for display; the movie has been corrected for drift with bilinear interpolation; top: front view, bottom: side view; scale bar, 5 µm). Top right: kymographs obtained along cilium encircled in images. Blue line in both kymographs represents the brightest trace in the side-view kymograph. Red line highlights how the slope of trace in the front-view kymograph deviates. Bottom: velocity as function of the position along the cilium (cilium base taken as reference) as measured from the front view (two-dimensional, grey) and from the combined front and side views (three dimensional, black). The error made in the front view only velocity determination is indicated in red. Dashed curves correspond to standard errors of the mean. (c) Three-dimensional structures of C. elegans phasmid neurons obtained from a single image employing depth-of-field extension. Top: side and front views extracted from image series in Visualization 3 (movie recorded at 5 frames per second; top: front view, bottom: side view; scale bar, 5 µm). Bottom: three-dimensional structure of phasmids and xy, xz and yz-projections. (d) MPoVM allows imaging spatially separated planes in the head of C. elegans. Top: images (extracted from Visualization 4; movie taken at 5 frames per second; scale bars, 10 µm) taken at different depths in the animal (front-view plane translated, side-view plane fixed). Bottom: schematic representation of the object planes imaged.]
3.2 Three-dimensional particle tracking

In a second set of experiments, we investigated moving objects that cannot be tracked using classical three-dimensional single-particle tracking, because of poor contrast and overlap. To this end, we imaged the motion of groups of fluorescently tagged kinesin motor proteins performing intraflagellar transport [25] (IFT) in C. elegans phasmid cilia, Fig. 3(b) and Visualization 2. Accurate measurement of velocities is crucial for understanding motor-protein cooperation in IFT, since cargoes are transported by three motors with distinct properties [26,27] (velocity, direction). So far, IFT velocities have only been measured in a single plane of observation. This approach might cause substantial projection artifacts, since cilia are not straight and hardly ever in focus over their full length. Current three-dimensional imaging techniques based on sample or laser scanning cannot keep up with kinesin's relatively fast motion (about 1 µm/s) [26,27]. MPoVM, which simultaneously images front and side views, is well suited to follow these groups of motors in three dimensions in real time, Fig. 3(b). From time series of these simultaneously recorded front and side views, kymographs can be generated in both views, which can be used to accurately determine velocities. In these experiments we noted that (two-dimensional) velocity measurements using single imaging planes, as would it be obtained with conventional epi-fluorescence microscopy, result in an underestimation of the real (three-dimensional) velocity of up to 30%, Fig. 3(b). This demonstrates that the extra dimension provided by MPoVM allows accurate quantification of real-time, three-dimensional motion.

3.3 Volumetric imaging of sparsely distributed objects with extended depth of field

As shown above, MPoVM allows imaging a sample simultaneously from different viewpoints. In this way, three-dimensional information can be obtained, but only in the overlap zone of both image planes. In many samples, the volume of interest is thicker than the above examples, and it would be advantageous to record this volume in real time. In the following we demonstrate that this can be done using MPoVM by extending the depths of field of both image planes, by moving the main objective 5 µm up and down at 30 Hz while imaging front and side views at 5 Hz. We applied this approach to the tail of a living C. elegans roundworm and were able to image a volume of approximately 10 x 50 x 5 µm³ (lateral x lateral x axial) at 5 Hz, which allowed localizing the animal's two phasmid cilia pairs simultaneously, Fig. 3(c) and Visualization 3. From a single image (containing both viewpoints) in the sequence, the accurate, three-dimensional structure of the phasmids could be obtained. Note that in situations with more densely distributed structures that overlap significantly in both views, the extraction of individual features would be very difficult. Therefore, we suggest to use this volumetric technique only to observe sparsely distributed structures.

3.4 Oblique imaging of spatially separated parts of the sample

In a final set of experiments, we demonstrated that MPoVM can be used to image two spatially separated parts of the sample at the same time. This can be very advantageous in applications like the recording of neuronal-network activity [28]. Using traditional microscopy methods this is difficult to achieve. MPoVM allows the simultaneous imaging of two separate object planes, which makes such an experiment straightforward, even when the locations are tens or hundreds of micrometers apart. To demonstrate this capability of MPoVM, we simultaneously imaged two planes in C. elegans: the side view was kept fixed in the animal, while the front view was translated over tens of micrometers along the axial axis, revealing different structures within the animal, Fig. 3(d) and Visualization 4.
4. Discussion

We have developed a microscopy method, MPoVM, that combines a micro-mirror in the sample and refocusing, allowing the simultaneous imaging of front and side views of an object. This allows the straightforward extraction of real-time, three-dimensional insights in dynamical processes, which is difficult to obtain with other conventional means. The time resolution of the approach is limited only by signal-to-noise ratio and the specification of the camera used. Under our conditions, i.e., a relatively sparse distribution of fluorophores, the axial resolution is about a factor of two better than that of wide-field fluorescence microscopy. In contrast to other approaches, MPoVM does not provide optical sectioning. For more dense fluorescent samples, overlap between different objects in the fields of view can occur, making it very difficult to distinguish them. For such samples, MPoVM is not the technique of choice. Since MPoVM has a larger field of view than methods employing multiple parallel planes, it can be used to image larger volumes when combined with extended depth of field, again only for sparse samples with limited overlap of objects in the fields of view. MPoVM is a relatively simple and cost-effective approach, not depending on expensive and vulnerable moving parts, that can be readily implemented on existing fluorescence microscopes. MPoVM can be extended with additional functionality, for example to optically extend the depth of field to up to tens of micrometers [29–32], or combined with light-sheet excitation and detection [33]. This latter approach, however, will substantially increase the complexity of the current setup. Furthermore, MPoVM is not limited to fluorescence microscopy and can in principle be used for bright-field microscopy, including phase-contrast methods, also opening up these approaches to dual plane oblique imaging.

5. Methods

5.1. Microscopy setup

The microscope was custom built using Thorlabs opto-mechanical parts. A sample was illuminated with 488 nm light from a Coherent Sapphire 488 LP laser. A Semrock Di01-R488/561 dichroic mirror was used to reflect the laser excitation into the objective. A 60X NA 1.20 water immersion UPlanSAPo Olympus objective was used as the main microscope objective, while the refocusing objective was a 40X NA 1.30 oil immersion CFI Plan Fluor Nikon objective. Flat mirrors, including the one facing the refocusing objective, were broadband dielectric BB1-E02 Thorlabs mirrors. The micro-mirror as well as the mirror facing the refocusing objective were mounted on piezo-driven linear stages, Newport AG-LS25. Thorlabs achromatic doublets lenses of different focal length were used. Fluorescence emitted by the sample was filtered by a Semrock FF03-525/50-25 Brightline band-pass filter. Images were captured with an Andor iXon Ultra 897 EMCCD camera, operated using Micro-Manager image-acquisition software (µManager, Micro-Manager 1.4, http://www.micro-manager.org).

5.2. Micro-mirror

Micro-mirrors were produced by replicating the Optometrics grooved beam divider 4-2460 (4 facet pairs per mm) in polydimethylsiloxane (Sylgard 184). It has been shown that this replication process is very accurate, yielding a surface roughness of only a few nanometers [34]; surface flatness is in most cases limited by the original optical component. After curing the PDMS was peeled from the structure and cut into small pieces, mounted on a thicker slab and then sputtered with 10 nm thick chromium and a 100 nm thick aluminum layer to create a reflective surface.
5.3. Three-dimensional localization from two views

Front and side views share the same y-axis, which was used as a reference. The position of the object along the x-axis was determined from the front view. The position along the x'-axis, perpendicular to the y-axis in the side view, is a linear combination of positions along the x- and z-axes, depending on the angle between front and side views. In our case, the side view was tilted 60 degrees with respect to the front view (along the y-axis): \( x' = x/2 - z \sqrt{3}/2 \).

5.4. C. elegans

The *C. elegans* strain expressing EGFP-tagged OSM-3 kinesin motor proteins [26] was a kind gift of Jonathan M. Scholey (University of California, Davis). Fluorescence imaging in living *C. elegans* was performed on adult worms (maintained at 20°C) in M9 buffer containing 5 mM levamisole (Sigma, Tetramisole hydrochloride, L9756).

6. Appendix: theoretical estimation of tracking error with micro-mirror imaging

Imaging through a micro-mirror is known to generate potential aberrations, as discussed by Berglund and coworkers [20]. In short, the cone of light emanating from a single particle harvested by a microscope objective in wide-field microscopy can be truncated when the image of the particle is observed through a micro-mirror, because of geometrical constraints, Fig. 4(a). As a result the image of the particle is affected, which can lead to an asymmetrical point-spread function (PSF), Fig. 2(a). These effects have been reported for mirrors at angles creating stronger aberrations than the ones used in our current study, but even in those more unfavorable conditions, aberrations have been claimed to be negligible in the vicinity of the mirror [12].

To estimate the potential tracking errors of MPoVM, we used an approach very similar to Berglund and coworkers [20]. A particle centered on the optical axis of a microscope objective and defocused at a distance \( z \) from the focal point generates an electrical field at the back focal plane of the objective that can be estimated with a scalar method as discussed before [26]; the amplitude of the electrical field is assumed to be constant, while the phase takes the following form: \( \Psi(\rho, \phi) = kn \sin \alpha \left(1/\sin^2 \alpha - \rho^2\right)^{1/2} \), where \( k \) is the vacuum wavenumber, \( n \) the refractive index of the immersion medium, \( \alpha \) the maximum aperture angle of the objective and \( \rho \) and \( \phi \) the normalized pupil radius and the azimuthal angle in the pupil plane, respectively. The formula takes into account the fact that the microscope objective follows Abbe’s conditions. Since the geometrical constraints of micro-mirror imaging limit the range of angle at which a ray can be reflected, the electrical field \( E \) at the back focal plane of the objective is \( E(\rho, \phi) = E_0 e^{i\Psi(\rho, \phi)} H(\rho, \phi) \), with \( E_0 \) the amplitude of the electrical field and \( H \) a function describing the geometrical constraints, i.e. \( H(\rho, \phi) = 0 \) or \( 1 \) depending on whether a ray can emerge or not from the micro-mirror, Fig. 4(b). The image of the particle on the camera plane is then obtained by Fourier transforming the amplitude of the electrical field in the back focal plane of the objective [20], from which a three-dimensional PSF can be calculated by simulating the image of a particle on multiple positions along the z axis. Note that in this theory, only the mirror geometry affects imaging, but not its size.

The simulated PSFs show an axial tilt that is hardly noticeable in the case of particles close to the micro-mirror and slight when moving away from the micro-mirror, Fig. 4(c), in agreement with our experimental observations. We estimated the tracking error using the micro-mirror image. The error is a function of the tilt angle and the axial spread of the PSF; the error is estimated by calculating the shift in centroid position at height \( z \), where the maximum intensity in the xy-plane is half the intensity when completely in focus, Fig. 4(d). The tracking error is less than 30 nm for a large portion of the space below the micro-mirror, which is of the order of the typical localization accuracy range reported when localizing a single molecule [35,36], Fig. 4(e). Therefore we believe these effects are not a problem for most applications.
Fig. 4. Theoretical estimation of tracking error with micro-mirror imaging. The conditions of the simulation correspond to the ones of the experiments, the mirror angle is 30° with respect to the horizontal and the objective numerical aperture is 1.20. (a) The mirror image of a particle is affected by micro-mirror geometry: the maximum angle at which a ray can emanate is limited and can in some case be lower than the microscope objective acceptance angle. In the figure, the maximum angle defined by the position of the particle with respect to the edge of the micro-mirror (here angle $\beta$) is lower than the objective acceptance angle $\alpha$. (b) The rays emanating from the micro-mirror, resulting from the mirror image of a particle, do not always fully cover the back focal plane of the objective. The geometrical constraint function $H$ defined in the text is 0 in the grey area and 1 in the white area; the case shown corresponds to the geometry of (a). O is the center of the back focal plane. (c) Examples of xz-projections of simulated PSFs of mirror images of particles close to the micro-mirror (top) and lower than the edge of the micro-mirror (bottom). The positions of the particles used for these simulations are indicated in the schematics (green dot, left). Scale bars, 1 µm. (d) Schematic indicating the definition of the tracking error estimate $\varepsilon$ in the x direction for localizing a particle using its mirror image. Scale bar, 1 µm. (e) Estimate of the position-dependent tracking error using the mirror image. The tracking error is estimated for mirror images emanating from the right-hand side reflector. The limit between two different grey areas corresponds to a line where the error is constant; highlighted are the lines for 5 nm, 30 nm and 100 nm tracking error respectively in solid, small dashed and large dashed lines. The cyan asterisk corresponds to the position of the bead used to generate the PSF in Fig. 1(c). The area encircled in cyan corresponds to the typical position of the animals used to generate Fig. 3.
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