Mesoscopic in vivo 3-D tracking of sparse cell populations using angular multiplexed optical projection tomography

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Abstract: We describe an angular multiplexed imaging technique for 3-D in vivo cell tracking of sparse cell distributions and optical projection tomography (OPT) with superior time-lapse resolution and a significantly reduced light dose compared to volumetric time-lapse techniques. We demonstrate that using dual axis OPT, where two images are acquired simultaneously at different projection angles, can enable localization and tracking of features in 3-D with a time resolution equal to the camera frame rate. This is achieved with a 200x reduction in light dose compared to an equivalent volumetric time-lapse single camera OPT acquisition with 200 projection angles. We demonstrate the application of this technique to mapping the 3-D neutrophil migration pattern observed over ~25.5 minutes in a live 2 day post-fertilisation transgenic LysC:GFP zebrafish embryo following a tail wound.

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OCIS codes: (170.2520) Fluorescence microscopy; (170.6900) Three-dimensional microscopy; (170.6920) Time-resolved imaging; (170.3010) Image reconstruction techniques.

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#226726 - $15.00 USD

Received 12 Nov 2014; revised 23 Dec 2014; accepted 25 Dec 2014; published 12 Mar 2015

(C) 2015 OSA | 1 Apr 2015 | Vol. 6, No. 4 | DOI:10.1364/BOE.6.001253 | BIOMEDICAL OPTICS EXPRESS 1253
1. Introduction

Understanding biological systems necessitates studying not only the spatial distribution of cells but also their temporal dynamics. The latter includes cell migration, which is crucial to studies of metastasis in cancer and to the recruitment of leukocytes toward sites of tissue damage in the inflammatory response. While cell migration studies can be undertaken in 3-D tissue culture models, it is often preferable to study the \textit{in vivo} migration of cells in disease models. Zebrafish larvae have emerged as a key model organism for inflammation studies due to their short reproduction cycle, optical clarity during embryogenesis, easy drug administration and the potential for manipulation using genetic and molecular approaches. In particular, the optical transparency of zebrafish allows visualization of physiological and pathological processes in the intact organism, which can provide insights into the global response of organisms to insults and disease. This has motivated two-dimensional [1–3] and three dimensional [4–7] imaging studies of \textit{in vivo} cell migration in sub regions within zebrafish using fluorescence microscopy but to date there has been no report of studies of 3-D time-lapse cell-tracking techniques throughout the whole volume of a fish.

To date, optical 3-D cell tracking has been implemented in microscopes that acquire 3-D volumetric acquisitions at each time point. Confocal/multiphoton laser scanning microscopes provide optical sectioning [4–6] that can be used to facilitate the acquisition of 3-D image z-stacks of fluorescently-labelled cells and this approach has been used to study neutrophils in zebrafish with high resolution during inflammation in a small region (e.g. tail wound model, ~100 µm field of view) [7]. However, most laser scanning microscopes are practically limited in penetration depth and field of view (~100’s µm) and they inherently exhibit anisotropic resolution. While they could, in principle, be extended to mesoscopic imaging of whole (~mm scale) samples, this would require high excitation powers and/or long image acquisition times. As an alternative, several “mesoscopic” (1-10 mm) 3-D imaging techniques have emerged that benefit from full-field image acquisition including optical projection tomography (OPT) [8] and light sheet microscopy (LSM) [9,10]. These techniques can be directly extended to 3-D volumetric imaging (i.e. acquisition of entire sample volume at each time point) and it is straightforward to realise 3-D time-lapse feature tracking by implementing the algorithm already developed for laser scanning microscopy [7]. Unfortunately the volumetric imaging entails acquiring a full stack of images for LSM or a full set of projections for OPT at each time point and to realize cellular resolution throughout a mm-scale volume requires >100 frames per volume. This limits the time lapse resolution and, for OPT, can critically compromise the 3-D reconstruction of features that move a significant distance during this volumetric acquisition time. Further, the acquisition of 100’s of images at each time point results in a significant light dose to the specimens throughout the time course, which may result in photobleaching and/or phototoxic effects that could compromise the \textit{in vivo} behavior under study.
To address this challenge, we propose an alternative approach to 3-D tracking that extends the approach of angular multiplexing OPT [11] to realize 3-D feature localization at the camera frame rate by simultaneous image acquisition at multiple different projection angles such that the position of features in 3-D can be estimated using triangulation at each time point without reconstructing the whole sample volume. This enables 3-D feature tracking with a time resolution equal to the delay between sequential projection acquisitions and a light dose for each time point given by the dose for a single image acquisition rather than for an entire volumetric acquisition. It also provides isotropic spatial resolution (unlike 3-D tracking implemented with confocal/multiphoton laser scanning microscopy or typical light sheet microscopy). When implemented during an OPT acquisition, the 3-D feature tracking can be superimposed on a time-integrated volumetric image of the sample. This is particularly convenient when tracking cell migration in a disease model such as zebrafish. We note that the sample rotation inherent in OPT removes ambiguity in the positions of features in the same plane (i.e. the same Z position in the coordinate system described in section 3) that remain stationary during time-lapse imaging or move in a correlated way over the acquisition time, for example when motion is instigated by a localized stimulus.

Here we demonstrate this approach by applying dual axis OPT (with simultaneous image acquisition at two orthogonal projection angles) to realize 3-D time-lapse tracking of neutrophils migrating within a LysC:GFP transgenic zebrafish embryo (2 days post-fertilisation) with a tracking time resolution of ~15 s.

2. Materials and data acquisition

2.1 Sample preparation

A transgenic mutant zebrafish line (LysC:GFP mitfa−/− roy−/−), which is transparent and expresses GFP in neutrophils, was used as an exemplar model for \textit{in vivo} time-lapse imaging of cell migration. Embryos were raised in embryo medium (dechlorinated system water containing 0.0003% (v/v) methylene blue) and at 2 days post-fertilisation (dpf) were embedded in 1% low melt point agarose, made from embryo media, containing 0.3 mM MS-222 (Sigma) as an anesthetic. After anesthetization, they were drawn into translucent FEP tubing (06406-60, Cole-Parmer Instrument Co. Ltd.) with a refractive index similar to that of water that can be used in an index matched chamber for imaging applications. The translucent FEP tubing had inner and outer diameters of 0.8 and 1.6 mm respectively. Agarose was added to increase the viscosity of the water and prevent movement of the anaesthetized zebrafish embryo. A tail injury was induced 30 minutes before imaging to provide an inflammatory stimulus.

2.2 Imaging system

The setup of the angular multiplexed OPT system was modified from the configuration described in [11] by focusing both of the orthogonal imaging arms onto the axis of rotation (see Fig. 1 inset). The experimental configuration of the dual image acquisition system is depicted in Fig. 1. In brief, the sample was mounted under a rotation stage (T-NM17A200, Zaber Technologies Inc.) and suspended in a water-filled cuvette to provide index matching. A commercial 25 mW laser at 473 nm (Blues, Cobolt Inc.) was used for wide-field excitation, incorporating a rotating diffuser to time-average speckle in the recorded images. The emitted fluorescence was imaged onto two CCD cameras (Clara, Andor Technology plc, 1040 × 1392, 6.45 µm pitch size, cooled to −20 °C) using two identical imaging systems (L1: 25 mm achromatic doublet lens, L2: 50 mm achromatic doublet lens and an adjustable aperture) at orthogonal projection angles via appropriate emission filters (520 ± 17.5 nm), producing a field-of-view of 3.35 × 4.49 mm² (× 2 magnification). The collection NA was adjusted using the apertures (AP) in the back focal planes of lenses L1, producing an effective collection NA of 0.033 with a depth of field of 0.78 mm to approximately match the inner diameter of the FEP tubing. The resulting in-focus lateral resolution was ~10 µm. An electronically controlled...
shutter (SH05 and apt-Solenoid controller, ThorLabs Inc.) was positioned directly after the excitation laser to prevent light exposure between projection acquisitions.

![Schematic of dual axis OPT set-up](image)

Fig. 1. Schematic of dual axis OPT set-up (inset shows the focusing of the orthogonal imaging systems to planes intersecting at the axis of rotation). EF – emission filter, AP – aperture, L1 and L2 – tube lens, FP – focal plane, $\phi$ – sample diameter, DOF – depth of field.

2.3 Acquisition settings and procedure

The acquisition settings and hardware were controlled using a program written in LabVIEW 2010 that was developed in-house. To realize simultaneous acquisition by the two CCD cameras, both cameras were set to external trigger mode to allow simultaneous triggering by a TTL signal from a DAQ board (USB-6008, National Instruments Corp). For a time-lapse acquisition, the excitation shutter was opened and the CCD cameras triggered by the signal generator. Once the CCD integration time was reached, the shutter was closed and the rotation stage switched to the next angular position while the CCD cameras were read out sequentially and the images were saved. This process was repeated after a user-defined delay time for the desired number of rotation steps.

For the demonstration presented here, 100 pairs of orthogonal projection images were acquired at 0.9° angular intervals every 15.3 s (0.3 s CCD integration time plus 15 s time-lapse delay), a typical time-lapse delay used for 2-D tracking of neutrophils in zebrafish. Thus the sample was rotated by a total of 90° during the acquisition of ~25.5 minutes, during which time the total exposure time of the zebrafish embryo was ~30 s. In this experiment a standard transmitted OPT data set was subsequently acquired at 0.9° angular steps over a full rotation (360°) to obtain a 3-D reconstruction of the zebrafish embryo structure.

3. Analysis methods

3.1 Cell detection and identification

The raw data from the orthogonal fluorescence projections (i.e. pairs of images acquired at 90° with respect to each other) defines the experimental coordinate system, $X,Y,Z$, where $Z$ is a common vertical coordinate (parallel to the axis of rotation) measured from the bottom row of the projection images, $X$ is the lateral coordinate measured from the left hand side of the projection image for one camera and $Y$ the lateral coordinate for the orthogonal camera. Over a full acquisition there are $k$ pairs of projection images from which we can determine
The coordinates of fluorescent objects (i.e. cells) in the $k^{th}$ image pair, through image segmentation and triangulation.

Fig. 2. (a,b) Simultaneously acquired orthogonal pair of fluorescence images of a 2 dpf LysC:GFP zebrafish to which the size-selective cell segmentation has been applied and (c) the corresponding transmitted light image for (b) showing cell localization within the zebrafish (only the tail is shown here). Scale bar 200 μm. The white interrupt on the scale bar indicates the cell localization accuracy (2 pixels). Inserts show magnified view.

Ideally, the fluorescent features (neutrophils expressing GFP in this demonstration), would present constant brightness across different time points. In practice, however, the measured fluorescence intensity is affected at different rotation angles by the light collection efficiency (which varies with distance to the focal plane), the spatial variation in excitation light and attenuation of both excitation and fluorescence light through scattering and absorption. In addition, cells typically display a varying fluorescence protein expression level from cell-to-cell and can stretch and change their shape significantly during locomotion. We therefore implemented a size-tuned, nonlinear top-hat function image processing algorithm in Matlab to identify objects within an expected size range [12,13]. The method is based on the ratiometric comparison of the average intensities between a given group of pixels and their surrounding area. In our implementation the sizes of these vicinities were set to 2 and 8 pixels respectively, where a pixel was 3.25 μm in width. The segmentation was followed by a morphological smoothing and size sieving, where the size of the segmented objects was limited to a minimum of 12 square pixels. In order to separate possible touching/overlapping cells, a watershed procedure was applied.

Figures 2(a), 2(b) show the size-selective cell segmentation applied to an orthogonal pair of source fluorescence images of the zebrafish embryo, while 2(c) shows the segmentation for 2(b) superimposed on the corresponding transmitted light image, confirming that localization is within the zebrafish. Following segmentation, the $(X,Z)$ and $(Y,Z)$ coordinates of the identified cells in each projection image pair were assigned as the centroid of their segmented areas. The same cells were identified in the projection image pairs by proximity of the common $Z$ coordinate (within a $Z$ tolerance of ± 3 pixels). The final assigned $Z$ coordinate for identified cells was the average from the projection pairs. This data was stored for each cell for further analysis.

3.2 Tracking algorithm

The first step towards quantitative analysis is to reconstruct the trajectories of the segmented cells from the measured projection coordinates. The general approach is to link every object at a give time-point to the ‘nearest’ object at the next time-point. Sophisticated methods may include position, brightness, size, shape, direction, velocity and other features for calculating ‘proximity’, but in this demonstration experiment we limit our algorithm to geometric distance only. We transform the list of $(X_k,Y_k,Z_k)_k$ coordinates at the $k^{th}$ time-point for all the
identified cells into the coordinate system for the $k^{th} + 1$ time-point using the transformation given by Eq. (1), where $\theta$ is the angular step between successive acquisitions and $X_{AoR}$, $Y_{AoR}$ are the lateral positions of the axis of rotation in the orthogonal images. These transformed coordinates, $(X_{k + 1}, Y_{k + 1}, Z_{k + 1})$, are compared to the coordinates identified at the $k^{th}$ + 1 time point, $(X_k + 1, Y_k + 1, Z_k + 1)_{k + 1}$, and cell coordinates within a radial distance of 6 pixels from each other are identified as the same cell. The coordinate data is then added to the track data for that cell, with the constraint that each cell can only be added to one track.

$$
\begin{align*}
X_{k+1} - X_{AoR} &= \begin{bmatrix} \cos \theta & -\sin \theta \\ \sin \theta & \cos \theta \end{bmatrix} \begin{bmatrix} X_k - X_{AoR} \\ Y_k - Y_{AoR} \end{bmatrix} \\
Y_{k+1} - Y_{AoR} &= \begin{bmatrix} \cos \theta & -\sin \theta \\ \sin \theta & \cos \theta \end{bmatrix} \begin{bmatrix} X_k - X_{AoR} \\ Y_k - Y_{AoR} \end{bmatrix} \\
Z_{k+1} &= (Z_k)_{k + 1}
\end{align*}
$$

(1)

If a corresponding cell cannot be successfully identified at the $k^{th} + 1$ time-point, then the algorithm compares the $k^{th}$ coordinates to coordinates at the $k^{th}$ + 2 time-point using the appropriate transformation. If a corresponding cell can still not be identified then the track data for that cell terminates at the $k^{th}$ time-point.

![Image](a) ![Image](b)

Fig. 3. (a) 3-D plot (Media 1) showing the neutrophil migration tracks over ~25.5 minutes with (b) corresponding migration mapping within the zebrafish (color used to differentiate tracks). Scale bar 200 $\mu$m. Scale and orientation of (a) and (b) are approximately equal.

Since this simple algorithm only compares neighboring time-points at each iteration, it can be initialized at any time-point in the data set and run both forwards (i.e. compare $k$ with $k + 1$ until the final time-point) and backwards with respect to acquisition order (compare $k$ with $k-1$ until the first time-point). To maximise the number of cells tracked across the complete time-lapse acquisition series, the data was reanalyzed starting from each of the 100 time-points. The identified tracks were compared, and broken tracks were connected if their ends were within 8 pixels. Finally, the cell tracks were converted to an object coordinate space, which is defined as the experimental coordinate space at the first time-point (i.e. $k = 1$).
3.4 Characterization of reconstructed tracks

After reconstructing the trajectories of the tracked cells, a variety of characteristics can be estimated, including the total trajectory length (i.e. the total distance traveled by a specific cell), the migration length with respect to the different axes, the net displacement traveled (i.e. the distance between the start and end points), as well as different velocity measures. As examples of track characterization, we calculated the average track speed (the total length of a track divided by the track’s time interval) and the directionality (the average cosine of the angle between two consecutive increments). The value of the directionality varies between 0 for completely disordered motion and 1 for unidirectional linear motion.

3.5 Software used

All algorithms, including OPT reconstruction, were implemented in Matlab. The Icy [14] “Spot detector” plug-in was used to benchmark the segmentation method, resulting in very similar performance. To represent the 3-D cell trajectories color-coded by speed and directionality in combination with the transmitted light volumetric reconstruction (see Fig. 3(b) and 5) images were produced using Voreen (http://voreen.uni-muenster.de/).

4. Results and discussions

A series of tracks for individual neutrophils in the fish tail were determined from the projection images, as described above, with the Z axis approximately aligned along the length of the fish. The total number of cells identified by the nonlinear top-hat image processing algorithm across all 200 projection images was ~8000, from which 82 tracks found to be suitable for quantification were identified. Figure 3(a) (Media 1) shows all the identified 3-D neutrophil migration tracks while 3(b) shows the corresponding migration mapping superimposed onto the volumetric reconstruction of the zebrafish embryo, which was obtained by performing a filtered back-projection reconstruction [15] on the acquired transmitted light OPT data. For the 82 tracks identified in this data set, 5 pairs of tracks had Z-coordinates within the Z-tolerance range (3 pixels) for 25% of the acquisition time, and among them 1 pair for 75% of the acquisition time. These extended periods of overlap in the Z-plane confirm the need for rotation during the acquisition procedure to remove ambiguity in the 3-D location. This becomes more important as the number of cells increases.

The tracks illustrate that angular multiplexing OPT can be employed to track mesoscopic 3-D features with a time resolution equal to the time between successive orthogonal projections (15.3 s in this demonstration) and at a fraction of the light dose required for volumetric imaging at each time-point. For example, the total exposure time, and therefore light dose, would have been 200 times greater if a volume per time-point was acquired (assuming 200 projections acquired over a 180° rotational scan) and the time-lapse resolution would have been limited to ~1 minute. This significantly reduced light dose enables more extended time-lapse in vivo tracking. We note that the time resolution and overall time-lapse of tracking can be adjusted within the constraints of the highest temporal resolution being limited by the single image acquisition time and the time required to move the sample to the next position. Figure 4 shows the individual cell migration with respect to the Z axis (i.e. approximately aligned along the zebrafish tail) during the tracking time.
Fig. 4. Individual cell migration paths along the Z axis (approximately aligned with the zebrafish tail) during the ~25.5 minutes time-lapse. Negative displacements indicate travel towards the tip of the tail. The red vertical scale bar indicates the resolution of the cell localization algorithm.

The vertical scale bar indicates the accuracy of the implemented cell segmentation and localization algorithm, so displacements at or below this scale may be artefacts of the analysis approach. The spatio-temporal dynamics of a cell population represents a rich data set that can be analysed with respect to a number of parameters according to the requirements of the biological experiment. Here we present exemplar readouts of 3-D cell tracks color-coded by average track speed Fig. 5(a) (Media 2) and average directionality Fig. 5(b) (Media 3) displayed in the context of the zebrafish volume.

Fig. 5. 3-D cell tracks color-coded by (a) average speed (Media 2) and (b) the average directionality (Media 3).
5. Conclusions

In conclusion, we have demonstrated a technique for mesoscopic 3-D cell tracking combined with OPT that utilizes multiplexed angular projections to realize a time lapse resolution determined by the delay between sequential projection images rather than the total volumetric acquisition time. This approach results in a significantly reduced light dose compared to time-lapse volumetric imaging and so is suitable for extended \textit{in vivo} studies. As an illustration of the technique, we demonstrated its application to map the 3-D migration of GFP-labelled neutrophils responding to a tail wound in a zebrafish embryo. This could be generally useful to study neutrophil migration in response to inflammatory stimuli and the technique can be applied to track any resolvable fluorescent feature moving in 3-D in semitransparent mesoscopic samples, including 3-D cell cultures and other model organisms such as \textit{C. elegans}. The tracking accuracy could be improved beyond this demonstration by employing more than two imaging projections and by utilizing a remote focal scanning OPT technique [16,17], which will enhance the resolution and the segmentation process. The procedure to identify the tracks of the migrating cells could be developed beyond the simple geometric proximity condition employed here by using more advanced algorithms to exploit additional parameters including, e.g. morphology or velocity. These could be directly implemented on a cell by cell basis as we have done here or optimization techniques that consider the track data set as a whole could be used.

Acknowledgments

The authors gratefully acknowledge funding from the Medical Research Council (MRC, MR/K011561/1), the Biotechnology and Biological Sciences Research Council (BBSRC, BB/L018039/1), the Wellcome Trust (Institutional Strategic Support Fund Networks of Excellence Award and WT 095931/Z/11/Z) and the European Foundation for the Study of Diabetes (EFSD). Lingling Chen acknowledges a former Lee Family Scholarship. Natalie Andrews acknowledges a studentship from the Institute of Chemical Biology EPSRC funded Doctoral Training Centre.