FULL ARTICLE

Three- and four-dimensional visualization of cell migration using optical coherence tomography

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Conventionally, cell chemotaxis is studied on two-dimensional (2D) transparent surfaces, due to limitations in optical and image data-collection techniques. However, surfaces that more closely mimic the natural environment of cells are often opaque. Optical coherence tomography (OCT) is a noninvasive label-free imaging technique, which offers the potential to visualize moving cells on opaque surfaces and in three dimensions (3D). Here, we demonstrate that OCT is an effective means of time-lapse videomicroscopy of Dictyostelium cells undergoing 3D (2D + time) cell migration on nitrocellulose substrates and 4D (3D + time) chemotaxis within low-density agarose gels. The generated image sequences are compatible with current computer-based image-analysis software for quantification of cell motility. This demonstrates the utility of OCT for cell tracking and analysis of cell chemotaxis in complex environments.

3D volume showing Dictyostelium discoideum cells within a 0.5% agarose gel on a base of 1.8% KK2 agar. The volume is 1300 μm × 1300 μm with a depth of 380 μm.

1. Introduction

Cell motility and chemotaxis are critical during embryonic and foetal development; and tissue growth and remodelling occur throughout life requiring regulated cell migration [1–4]. In adult health, related processes are an important aspect of new clinical technologies for tissue engineering, repair and replacement of damaged organs [4–7]. Conventional studies of cell migration have largely relied on the
use of 2D monolayer cultures on transparent surfaces, however it is questionable how well these relate to in-vivo processes [8, 9]. Control of cell behavior and morphology requires novel physical and chemical features incompatible with existing visualization techniques (e.g. substrates may be opaque) [10–16]. Proper formation of tissue requires 3D interaction between different cell types and mechanical load [3, 4, 7, 15–17]. Therefore, cells need to be grown within 3D matrices comprised of novel biomaterials that mimic in-vivo scaffolds [16, 18, 19]. A current major problem is how to analyze the morphology and behavior of cells cultured on nontransparent 2D surfaces and in 3D [3–5, 9, 13, 16, 20]. This information will provide insight into in-vivo cell migration and assist identification of required cell types within a 3D construct for transplantation [9, 16, 18, 21].

Cell behavior and phenotype have been shown to differ in 2D and 3D environments [22]. Although valuable insights into cell migration have been gained from 2D studies into cell movement, cells in vivo undergo morphogenesis and migrate in 3D. A complete understanding of cell behavior therefore necessitates noninvasive monitoring of cells in 3D over time [4, 14, 20]. Such studies of chemotaxis in 3D time-lapse can also aid the understanding of many important in-vivo processes, for example wound healing and function of the immune system; and offer insight into disease states involving aberrant cell migration such as cancer metastasis [2–4, 17].

Conventional microscopy techniques used in the study of cell migration, for example phase contrast or differential interference contrast (DIC) microscopy, do not allow visualization of cells on opaque surfaces or within scattering tissue and media [12, 14, 23]. Fluorescent confocal and multiphoton microscopy, commonly used for analysis of cell–substrate interactions and 3D cell behavior, can penetrate only about 300 μm into tissue and the exogenous fluorophores used may interfere with the biological processes taking place within the cell [2, 5, 16, 20, 24]. Additionally high laser intensities used in fluorescence imaging can cause photodamage to living tissue [5, 20, 24].

The noninvasive interferometric imaging technology of frequency-domain optical coherence tomography (OCT) overcomes these problems [2, 5, 16, 20, 25–27]. In OCT, refractive-index variations caused by membranes or vacuoles of cells eliminates the need for staining and sample processing, reducing artefacts and increasing cell viability [2, 5]. This is of particular significance when imaging over long periods of time. OCT is commonly performed at 800 nm, a wavelength largely transparent to water (the major component of tissue) giving a high penetration and visualization depth of up to 1–2 mm in scattering tissue [24, 25, 28]. Due to the interferometric determination of depth, OCT permits suppression of photons that have been multiply scattered allowing penetration to greater depths into scattering tissue than other imaging methods [24, 25]. Contrary to many other imaging techniques, axial resolution is dependent on the center wavelength and the optical bandwidth of the employed light source, and not on the numerical aperture of the objective [27]. OCT therefore enables high axial resolution of up to 1 μm using state-of-the-art technology [25, 29]. Transverse resolution, in common with other imaging techniques, is limited by the numerical aperture and can reach 1–2 μm with high NA objectives although resolutions of ~3–5 μm are often used in order to maintain a larger depth of field [24]. Due to the ability to acquire complete depth profiles in a single measurement, most recent developments in OCT detection techniques have enabled data acquisition rates of 20000–160000 samples per second [30]. These properties make OCT a potentially useful technique for investigating dynamic cell behavior on and within opaque materials.

The social amoeba Dictyostelium discoideum is a unicellular amoeba that migrates by extending pseudopodia in the desired direction of travel [31–34]. When starved of nutrients, it enters a developmental programme that culminates in the formation of multicellular structures of ~10000 cells [35–37]. Cells aggregate via a process of chemotaxis; one cell begins to emit pulses of cAMP, attracting other cells, which also propagate the cAMP signal [8, 34, 35]. This makes it an ideal system for the study of chemotaxis [38]. In this paper, we demonstrate that OCT can be used to follow chemotaxis of D. discoideum on opaque nitrocellulose membranes and within 3D gels over time.

2. Experimental

2.1 Cell culture

AX2, an axenic strain of D. discoideum, was grown in suspension culture in Axenic medium. Cells were collected by centrifugation in the late exponential growth phase and washed three times in KK2 buffer solution. For the 2D time-lapse experiments, cells were plated at a concentration of 10^7 cells on a 0.45 μm nitrocellulose filter (Millipore) or the equivalent area on 1.8% nonnutrient agar, (Figure 1) and developed for 8 h. Cells were then imaged at 30 s intervals for 13 min. For the 3D time-lapse experiments, 1.6 x 10^7 cells were suspended in 2 ml of 0.5% (w/v) low melting point agarose (Sigma-Aldrich) containing KK2, supported on a base of 1.8% agar. After 8 h, cells were exposed to a cAMP gradient by placement of a small droplet on the agarose surface that contains 1 μM cAMP in 1% agarose, and bro-
mophenol blue as a location marker. Cells undergoing chemotaxis were recorded in time-lapse with images every 120 s for 2 h.

2.2 Optical coherence microscopy

The OCT system (Table 1) is a fiber-based ultrahigh-resolution spectrometer-based frequency-domain system built using FiberCore SM750 single-mode fibers with a cut-off wavelength of ~650 nm. A Ti: sapphire femtosource integral OCT (Femtolasers Produktions GmbH, Austria) laser was employed as the broad bandwidth optical source. The sample arm consisted of a microscope with a galvanometric x/y scanner controlled by an FPGA (field-programmable gate array) and an objective of 10 mm working distance and ~0.1 numerical aperture. A movable stage equipped with a retro-reflector allowed adjustment of the reference delay and an attenuator was used to maintain the overall signal just below the saturation level of the spectrometer (incorporating an ATMEL AViiVA M2 CL 2048 pixel CCD) as this is dependent on the exposure time of the array. The FPGA was integrated with a Full-CameraLink capable frame grabber with a data transfer rate of up to 640 MB/s to the host PC. A Labview (National Instruments, USA) interface was used to control the FPGA, the real-time display and the data storage.

Table 1  OCM specifications.

<table>
<thead>
<tr>
<th>Specification</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central wavelength</td>
<td>800 nm</td>
</tr>
<tr>
<td>Bandwidth</td>
<td>120 nm</td>
</tr>
<tr>
<td>Power on sample</td>
<td>&lt;2 mW</td>
</tr>
<tr>
<td>Scanning speed</td>
<td>20 000 Hz</td>
</tr>
<tr>
<td>Axial resolution (measured)</td>
<td>&lt;4 μm</td>
</tr>
<tr>
<td>Transverse resolution (measured)</td>
<td>4 μm</td>
</tr>
<tr>
<td>Signal to noise ratio</td>
<td>90–95 dB</td>
</tr>
</tbody>
</table>

2.3 Data acquisition and processing

Volumes were collected in stacks of tomograms (typically consisting of 256 x 256 pixels with 1024 pixels in depth). Conversion to the spatial domain was performed by Matlab software that also corrected for dispersion, spectral shape and nonlinearity of the camera [39]. The volume stack was further corrected for jitter and speckle was suppressed by nonlinear filtering in ImageJ (NIH, USA).

2.4 Quantitative analysis

Detailed computer-assisted reconstruction and quantification of cell locomotion was accomplished using 2D-DIAS (Dynamic Image Analysis System) software (Soll Technologies Inc, Iowa, USA) [31, 32]. Outlines were traced around the perimeter of the chosen migrating cells from which 2D-DIAS generated a mathematical model and computed various motility parameters [31, 32]. This numerical information about cell behavior can be compared across different conditions and substrates to enable analysis of the effects of certain factors on cell morphogenesis and chemotaxis.

3. Results and discussion

3.1 OCT time-lapse of moving cells

Chemotaxis of *D. discoideum* is most often carried out with isolated cells plated in a gradient of cAMP on a glass microscope slide [8, 31]. However, Dictyostelium aggregation and subsequent development actually involves large numbers of cells plated on substrates such as non-nutrient agar or 0.45 μm pore...
nitrocellulose filters (Figure 1) [33, 40]. Although it is possible to track chemotaxis in cells on agar, this is rarely done. Furthermore, it is not possible to visualize cells directly on nitrocellulose. To explore the utility of OCT for imaging cell behavior, we examined migrating cells on both substrates.

Images of cells plated on 1.8% KK2 agar were taken at 30 s intervals for 13 min. Each scan volume was 256 x 256 x 1024 voxels, taking less than 10 s to acquire. To obtain the images seen in Figure 2, the data were restricted to a depth of 15 pixels (4 mm) incorporating the surface of the substratum and the cells. This depth was summed and rendered to form an en-face image for each time point (Figure 2(A)), which were combined to form time-lapse movies (see movies in supporting information). Color coding of individual time points enables qualitative evaluation of cell movements (Figure 2(B)). Individual cells were traced and tracked over time using 2D-DIAS, a computer-assisted cell-tracking program (Figure 2(C)). The speed of one representative cell was 6.5 mm per minute with a directionality (D-measure of linearity, calculated from the net path length divided by the total path length.) of 0.67. These movements compared well with previous data for wild-type cells, although cell movement may be slightly slower than the average 10 μm per minute seen on glass [8]. This demonstrated that OCT can be used to visualize and analyze cell movement.

3.2 Visualization of moving cells on a solid surface

An alternative substratum for development of *D. discoideum* is a 0.45 μm nitrocellulose filter. It is not possible to image cells developing on these filters with conventional transmission microscopy as light cannot penetrate through them. Figure 3 shows that using OCT, which is based on reflected light, it is possible to achieve cellular resolution on these opaque substrates and follow the development of *D. discoideum* in time lapse.

OCT en-face images can be magnified by scanning a smaller area (Figure 4). This increases the sampling density while maintaining the number of voxels and image acquisition time. In Figure 4 the cell outline is visible, enabling changes in morphology and cell polarization to be seen. Therefore, high-resolution information on cell behavior can be detected.

As seen on the agar substrate, individual cells moved in a relatively straight line (D = 0.73), but aggregated at a slightly slower speed than cells plated
In addition to cells, OCT revealed surface features of the substrate, showing that nitrocellulose has a rougher surface than agar. This means that OCT would be a good method to relate cell behavior to surface structures, and would facilitate investigation of cell interaction with complex and novel materials.

### 3.3 4D visualization of moving cells

To observe *D. discoideum* cells undergoing chemotaxis in a 3D environment, cells were suspended with even distribution in 0.5% low melting point agarose, supported on an agar base layer. Cells were allowed to develop for 8 h before cAMP stimulation. Agarose is relatively transparent to OCT at 800 nm, yet provides a solid structure through which cells are able to migrate. A chemoattractant was introduced by plating a drop of cAMP in agar on the agarose. cAMP diffusion into the agarose established a chemottractant gradient.

Volumetric images of cells within a 3D agarose construct (800 × 800 × 1024 voxels) were captured every 120 s over 120 min and are presented in Figures 5 and 6. Each volume took approximately 35 s to save and store. A 3D image showing single cells and multicellular aggregates suspended within the 0.5% agarose is shown in Figure 5(C). As cells have been developing for 8 h, by initiation of the image-recording session they have already begun to stream along and underneath the surface of the agarose. An en-face image (Figure 5(B)) of cells streaming at the top of the agarose and the cross-sectional image (Figure 5(D)) of cellular movement up towards the surface of the agarose indicate the total movement of the cells throughout the entire time course of the experiment. The approximate planes from which these images have been taken are indicated in Figure 5(C).

The paths taken by the migrating cells can be seen in Figures 6(A–D) for short (0–20 min) and long (0–120 min) time courses. The cross section of the short time course (Figure 6(B)) shows the gradual movement of cells and multicellular aggregates through the agarose towards the cAMP source. In Figure 6(A), at same time points in the en-face plane, cells can be seen joining streams and moving in a coordinated manner. This potentially simulates cellular movement in tissues [9]. Over the entire time-lapse experiment (Figures 6(C) and (D)) it can be seen that the cells move through the agarose towards the chemoattractant and come together, so that by the final time point there are hardly any cells or multicellular aggregates.
aggregates remaining that are not a part of the large multicellular mound. OCT can therefore offer interesting insights into the manner in which cells move towards a chemoattractant within a 3D matrix.

4. Conclusions

For ease of visualization, cells are most commonly grown on transparent substrates in 2D monolayers. This, however, differs substantially from the conditions they experience in their natural environment. *Dictyostelium discoideum* live at the air/water interface in soil and so are commonly developed on nitrocellulose filters (an air/water interface), as this simulates natural development. As these filters are opaque, cell migration during development cannot be imaged noninvasively by conventional means and cell-migration assays are usually performed on glass surfaces. As demonstrated here, OCT can noninvasively image in environments that are inaccessible to other methods. The images generated are compatible with existing image-analysis software (e.g. 2D-DIAS), which can be used to quantify the effects of different substrate structure and functionalization on cell locomotion. The same approach would allow cell migration to be visualized on engineered opaque biomaterials.

Recent developments in the field of tissue engineering have enabled cells to be grown in 3D organotypic cultures, which more closely resemble the complex 3D environment of the body than classical 2D monolayer culture. These cultures can enable insight into how cells behave in vivo as well as allowing cells required for transplantation to be grown in a more anatomically comparable environment. Tissue engineering within these 3D constructs requires a noninvasive imaging modality able to penetrate scattering media. OCT is emerging as a useful technique that is able to provide cellular resolution imaging within 3D scaffolds. This result demonstrates the potential of OCT to probe 3D environments.

With this experimental setup, penetration into the 3D construct is limited to approximately 400 μm by the depth of focus of the objective. This can be increased by use of an objective with a smaller numerical aperture. It is therefore possible to increase penetration depth at the cost of reducing transversal resolution. At reduced resolution, cells can be imaged as moving points, which can still yield information on speed and directionality of migration. Imaging under the same experimental conditions using a combination of objectives with different numerical apertures could be a useful way to obtain information on the character of cell migration at deeper levels of tissue while allowing cellular-level resolution imaging in the more superficial layers of the 3D environment. Alternatively, the interferometric synthetic aperture microscopy (ISAM) approach, which corrects for depth-dependent defocus, could be implemented and enlarge the visible depth [41].

Studies using the demonstrated technique will enable a greater understanding of the mechanisms of cell aggregation and morphogenesis and provide new techniques to monitor cell behavior for tissue engineering.
Figure 5 (online color at: www.biophotonics-journal.org)
(A) Schematic of 4D (3D+time) experimental set up. Cells were suspended within a 0.5% agarose solution on a base of 1.8% agar. A 100 µl spot of the chemoattractant cAMP was spotted onto the surface of the agarose to attract the cells. (B) En-face OCT images with time points (0–120 min) summed together showing streaming cell movement on the top of the 0.5% agarose. Streaming occurs when single cells come together, forming multicellular structures and all move together towards the source of the chemoattractant. Two streams can be seen in this image due to the fact that the cells forming the second stream are outside the area of effect of the chemoattractant and so have begun to spontaneously develop. (C) 3D volume (generated with Maxon Cinema 4D) showing the cells suspended in the 0.5% agarose gel at 0 min. The approximate location of the cAMP source is indicated with a yellow arrow. (D) Cross-sectional sum of B-scans (from the middle of the stack) of all time points (0–120 min) showing cells moving together and up to the surface of the 0.5% agarose. The scale bar is 100 µm.

Figure 6 (online color at: www.biophotonics-journal.org)
(A) Superimposed color-coded en-face OCT image of cells on the agarose construct, from summing slices over ~30 µm depth, blue – 0 min, red – 10 min and yellow – 20 min. (B) Superimposed color-coded B scan (cross section) of the agarose construct blue – 0 min, red – 10 min and yellow – 20 min. (C) Superimposed color-coded en-face view of cells on the agarose construct, from summing slices over ~30 µm depth, blue – 0 min, red – 60 min and yellow – 120 min showing streaming cell movement on the top of the 0.5% agarose. (C) Superimposed color-coded en-face view of cells on the agarose construct, blue – 0 min, red – 10 min and yellow – 20 min. (D) Superimposed color-coded cross section of the agarose construct blue – 0 min, red – 60 min and yellow – 120 min. Scale bars are 100 µm. Features constant throughout the time series appear white. Movies of the entire sequence of images in both the cross-sectional and en-face views rendered from OCT volumetric images can be seen online as supporting information (movies 4 and 5).
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References

APPENDIX

Supporting Information (available online)

Movie 1 [KK2 agar 2D video]
Cells are shown moving on KK2 agar in the en face plane rendered from OCT volumetric images. The frame rate is 5 fps and frames were acquired every 30 seconds.

Movie 2 [Nitrocellulose filter 2D video]
Cells are shown moving on a nitrocellulose filter in the en face plane rendered from OCT volumetric images. The frame rate is 5 fps and frames were acquired every 30 seconds.

Movie 3 [Nitrocellulose filter zoomed in 2D video]
Cells are shown moving on a nitrocellulose filter in the en face plane rendered from OCT volumetric images. The frame rate is 5 fps and frames were acquired every 30 seconds.

Movie 4 [En-face 3D video]
Cells are shown moving in 3D over the course of 120 minutes in the en face plane rendered from OCT volumetric images. The frame rate is 5 fps and frames are 10 minutes apart.

Movie 5 [B-scan 3D video]
The multimedia video shows the sequence of cell migration in its entirety in cross-section. Cells can be seen moving together and up to the surface of the 0.5% agarose. The frame rate is 5 fps and frames were acquired every 120 seconds.

Movie 6 [3D volume]
Rotating image from the cross-section to the top-down view showing the 3D structure of the cells within the agarose at 20 minutes after the chemoattractant was added. At this time point the cells have begun to clump together at the top right of the picture but individual cells can still be seen.