Cell motility studies using digital holographic microscopy

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1. Introduction

1.1 Cell movement

Studies of cell movement have many applications, such as invasion of malignant cells into healthy tissue and leukocyte migration into inflammatory sites. Cultured adherent cells usually move over their substrate in a crawling motion that varies greatly between cultures due to factors such as cell type, malignancy, mutations, confluence, substrate, or external chemical or mechanical stimuli. Cell movement can be divided into random movement (motility), and directional movement (migration). Different cell types may be characterized by their speed of movement. E.g. human fibroblasts move very slowly (12 – 60 µm/h) compared to neutrophils, which are the fastest moving leukocytes (900 – 1200 µm/h) [1].

1.2 Methods to study cell population movements

There are several well known methods to study cell movement, but they have mainly been directed at migration studies. Filter assays belong to one prevalent category of migration assays. The first, and most well-known, is the Boyden chamber [2], but there are also the later developed Zigmond and Dunn chambers [1]. The chamber assays are used to measure cell migration over a membrane in response to chemoattractants. The disadvantage of filter assays, is that they are very specialized, requiring the cells to migrate through both a matrix and the pores of a filter. This limits the choice of cells as very few cell lines are able to migrate through both matrix and filter.

In the scratch-wound, or wound healing, assay, a lane is scratched through cells in a confluent culture, thus creating an artificial “wound”. Cells will then move into the lane and repopulate it [3-5]. The disadvantage of the scratch-wound assay is that the result is a combination of proliferation and migration [1].

1.3 The study of single cell movement

The techniques described above focus on studying the average movement of large groups of cells. If possible, single cell studies should be performed as well as bulk studies. E.g. when studying population dynamics of carcinogenic cell types, the tissue usually consists of a mixture of malignant and benign cells. The distinction is rarely clear, but highly tumorigenic cells have earlier been shown to move faster than non-tumorigenic cells [6, 7], making it essential to follow both single cells and populations. Single cells can be studied using time-lapse imaging, often with fluorescent markers. Fluorescent imaging has two disadvantages. It can not be said to be non-disturbing, and the imaging time is limited by the bleaching of the fluorescent marker. Fluorescent imaging, phase contrast imaging and many other kinds of traditional imaging techniques, have a limited z-resolution and the sample often needs to be re-focused during imaging, due to focus drifting caused by thermal effects or mechanical instability. Also, no quantitative data can be extracted from phase contrast images.

1.4 Cell imaging and digital holography

The disadvantages of existing methods to study cell movement may be overcome by the use of digital holography. Reconstruction of a hologram by computer was first proposed by Goodman and Laurence [8] and by Kronrod et al. [9]. Digital holography is a full-field imaging technique that provides quantitative phase images from a single hologram. Since focusing adjustments may be computerized or adjusted afterwards, the common problem with drifting of focus in time-lapse photography is circumvented [10]. Furthermore, the interval with which images are recorded is limited only by the speed of the imaging sensor. The imaging is non-destructive and uses variations in the optical path length as a natural contrast agent. This eliminates the need for invasive contrast agents and makes it possible to study a sample for long periods of time with short interval between exposures, without reduced picture quality due to bleaching. The quantitative phase images obtained using digital holography are from a mathematical and image analytical point of view similar to those from a standard epifluorescence microscope. In many cases the same image analyzing algorithms can be used, such as watershed segmentation or active contour algorithms [11, 12]. The image contrast when
using digital holography is directly related to cellular dry mass making digital holography a more direct approach to study cells, if however not as versatile as fluorescent imaging.

1.5 How does digital holography work?

The human eye can record two properties of light; the intensity of light that corresponds to the amplitude, and the colour that corresponds to the wavelength of the light. However, when we using digital holography, we record a property of light that we can not see; the phase. To be able to detect and record the phase, all light waves emanating from the light source must be in phase, \textit{i.e.} the light source must be coherent. A laser is an example of a coherent light source, and our sun is an example of the opposite, an incoherent light source. The image of a hologram appears to be three dimensional, either inside or on top of the actual image. Since the phase of the light recorded in the hologram tells us how long the light has been gone, it also tells us how far away the object is. This causes the 3-D effect. One way of recording a hologram is to allow two identical beams of light to travel two paths of equal length, but only allow one of the beams to pass through an object. When the object blocks the path of one of the beams, thus delaying it, the light beam arrives at the detector a little late. The phase will then be a little different than that of the other beam, a difference called a phase shift. This causes an interference pattern. By studying the interference pattern of the arriving wave, we can calculate what the blocking object looks like.

In traditional optical microscopy, only the intensity of light (\textit{i.e.} the amplitude) is recorded. The microscope then measures how much light has been absorbed on the way. When imaging very transparent objects, the amount of light absorbed may be too low to be measurable. However, by measuring the phase shift of the light, very transparent objects can suddenly be made visible. In addition to this, a digital holography image contains information that can be quantified.

1.6 This project

The aim of this project was to develop a semi-automatic method to measure cell motility over time using undisturbed cells growing in conventional cell culture vials. We used digital holography in combination with computerized analysis of the resulting images. This allowed us to study the cells without disturbing them. In this study we show that cell movement data obtained by digital holography can be correlated to additional information, such as level of confluence, cell average optical thickness, cell area and cell volume [13-16].

2. Materials and methods

2.1 Cell lines and culture

Cell growth medium components were purchased from Biochrom Ag, Berlin, Germany and cell culture plastics were purchased from Nunc, Roskilde, Denmark unless otherwise stated.

The mouse subcutaneous connective tissue cell line, L929 (American Type Culture Collection, Manassas, VA, USA, ATCC Number: CCL-1) was grown in RPMI 1640 supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin and 10% fetal calf serum. L929 cells were subcultured once a week with medium changes every 2 to 3 days.

A cell line derived from human breast cancer lymph node metastasis, L56Br-C1 [17] , was grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 1 mM non-essential amino acids, 50 U/ml penicillin, 50 µg/ml streptomycin, 10 µg/ml insulin and 1 mM sodium pyruvate, and was subcultured once a week with medium changes every 2 to 3 days.

A human epithelial mammary gland cell line from a patient with adenocarcinoma, MDA-MB-231 (American Type Culture Collection, Manassas, VA, USA, ATCC Number: HTB-26) was grown in RPMI 1640 supplemented with 10% fetal calf serum, 1 mM non-essential amino acids, 50 U/ml penicillin, 50 µg/ml streptomycin and 1 mM sodium pyruvate. It was sub-cultured once a week with medium changes every 2 to 3 days.

For time-lapse experiments cells were seeded in 25 cm² cell culture flasks at different seeding densities. Variations in cell seeding density occurred in order for desirable confluenes to be obtained. Unless otherwise stated, the experiments were performed on cell populations with 10-20% confluence.

2.2 HoloMonitor™ M2

The instrument used was a Holomonitor™ M2 (Phase Holographic Imaging AB, Lund, Sweden), which integrates a phase contrast microscope with a digital holographic function. The laser used for the digital holography is a 0.8 mW HeNe laser at 633 nm. Both the reference beam and the object beam are filtered and made parallel using a beam expander with a pinhole. The light from the object beam passes through the sample and the light is collected onto a CCD camera using a standard microscope objective. The interference pattern on the camera is recorded. For each sample, three images were captured showing the interference pattern, the object light and the reference light. The image of the reference light and the object light were then subtracted from the image of the interference pattern.
2.3 Motility assay

A 25 cm$^2$ cell culture flask was placed on the digital holographic cell analyzer objective table on a warming plate (LRI Instrument AB, Lund, Sweden). Pictures were taken every 15 minutes. Calibration of pixel to micrometer scale was done using a ruler (Graticules LTD., Tonbridge, Kent, England).

2.4 Image reconstruction

After completion of the time-lapse, the images were processed and reconstructed by a computer algorithm [18]. Reconstructed phase images provided the starting point for further image analysis (Fig. 1). For time lapse analysis, single cells were detected in each image of a sequence and a two dimensional cross correlation of two successive images was performed. The movement of the cell from one image to next was measured using a correlation coefficient between each pair of imaged cells. The movement for each cell was tracked individually throughout the entire series, both relative to the first image of the series and to the previous image (Fig. 2). We used the program Celltrack (by Sven-Göran Pettersson) to follow the cells.

Figure 1. An example of reconstructed digital holographic images showing an area containing L929 cells over a period of 12.5 hours. Image A represents $t=0$ with each following image representing the status of the area 2.5 h after the previous image, ending with image F at $t=12.5$ h. Scale bar represents 20 µm.

Figure 2. A reconstructed digital holographic phase image showing the tracking of L929 cells for 14 hours. A. The tracking of a cell population. B. A 3-D rendering showing the tracking of a single L929 cell. The images were captured and the cells were analyzed at 10-20% confluence. Scale bar in A represents 50µm.

When recording both the direction and the distance moved the average of the total cell population in each image was zero or very close to zero, indicating that random movement was indeed measured. Throughout this study, the motility for each cell was measured as the sum of the recorded distance moved between each captured image. The direction moved was thus not accounted for.
Cell thickness was calculated from the phase shift assuming an average cell refractive index of 1.38 [19]. Thus any value of thickness presented is calculated regardless of any variation in refractive index between cell lines. The analysis of individual cell movement was done with an image spatial resolution of 1.4 µm with an inaccuracy of ±0.7 µm/image and 0.44 µm/hour. Cells with high and low motility are referred to as fast and slow cells, respectively.

3. Results

3.1 Average speed of movement

Three different cell lines were analyzed. All cell lines moved randomly through the culture. We show the results for L929 only (Fig. 2). L929 cells moved with an average speed of 4 µm/hour, L56Br-C1 cells with 1µm/hour and MDA-MB-231 cells with 3 µm/hour. The average speed was higher at the beginning of the experiment than later in the experiment (Fig. 3). The average values were consistently higher than the median values, indicating that a major part of the population moved slowly, while a smaller part of the population moved fast. This was confirmed when the motility of individual cells was plotted (Fig. 4). For all cells, the slowest 10% had motilities below the level of detection. For L929, the fastest 10% of the cells moved with 9 µm/hour. For L56Br-C1, the fastest 10% of the cells moved with 6 µm/hour. For MDA-MB-231, the fastest 10% of the cells moved with 12 µm/hour.

3.2 Effects of confluence on speed

We compared the speed of movement at different confluences for L929 cells. When the confluence was low, the average speed tended to be higher and when the confluence was high, the average speed tended to be lower, although the results are not clear-cut (Fig. 5). This effect is previously well-documented but detected with other methods [20]. The proportion of cells moving faster than 1.4 µm in each population does not seem to be affected by the confluence (results not shown).

3.3 Speed correlated to cell size, thickness, and volume

The cell motility can be connected to the size, thickness or volume of the cells. It is interesting to note that there were distinct values for average cell motility correlated to average cell area for each cell line (Fig. 6A). The results from the same cell line tended to cluster together indicating that L929 cells were larger and moved further than L56Br-C1 cells. MDA-MB-231 cells were smaller than L929 cells, but moved with similar speed. The average cell thickness did not vary as much as the cell area, and the cell line correlation was less clear (Fig. 6B). There were distinct cell line-correlated values for average cell motility correlated to average cell volume (Fig. 6C). The results from the same cell line clearly clustered together, showing that L929 cells had a larger volume and moved further than both L56Br-C1 and MDA-MB-231 cells. MDA-MB-231 cells had a smaller volume than both L929 and L56Br-C1 cells.
When comparing the 10% fastest moving cells in a population with the 10% slowest moving cells, the cells that moved further always had larger volumes than the slow cells (Table 1). When comparing cell motility correlation with the average cell area and cell volume in the populations (Fig. 6), both the fastest and the slowest group was smaller than the average for L929 cells, but larger than average for L56Br-C1 cells.

**Figure 3.** The average speed of movement of L929, L56Br-C1 and MDA-MB-231 cells over time. Images were captured and cells were analyzed at 10-20% confluence using digital holography microscopy. The experiments were repeated at least three times with at least 150 analyzed cells. Filled symbols represent median values while open symbols represent average values. A: L929 cells, B: L56Br-C1 cells, C: MDA-MB-231 cells. Bars represent ±SEM. Where the bars are not visible they are hidden behind the symbols.

**Table 1.** Showing the relative area, thickness and volume of the 10% slowest cells compared to the 10% fastest cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Relative Area</th>
<th>Relative Thickness</th>
<th>Relative Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>L929</td>
<td>100%</td>
<td>81%</td>
<td>80%</td>
</tr>
<tr>
<td>MDA-MB 231</td>
<td>88%</td>
<td>67%</td>
<td>59%</td>
</tr>
<tr>
<td>L56Br-C1</td>
<td>95%</td>
<td>101%</td>
<td>94%</td>
</tr>
</tbody>
</table>
4. Discussion

The primary aim of this study was to develop a simple method to measure cell motility. We aimed to make the method useful for most cell lines, and to make it simple enough to perform without specialist knowledge. Time-lapse studies using digital holography has been used previously for automatic tracking of moving cells in two and three dimensions with cells growing in a collagen tissue model [21]. The method we have developed allows for cell motility studies on adherent cells that are still in their cell culture vials, including cell culture flasks and cell culture plates up to 24 holes per plate. During this study we observed the motility of a small area of the cells in a T25 cell flask. The area is defined by the camera field of view (approximately 0.5 mm$^2$) and is in this report referred to as the population, but it is not in any way physically separated from the rest of the cells in the flask.

The results show that using digital holography in combination with our program Celltrack, we can follow a population of cells, and we can study single cells within that population. The results also show that we can correlate cell movement with the level of confluence in the cultures, cell area, cell optical thickness and cell volume. In all, digital holography proved suitable for a cell movement assay.

The tracking of cells using digital holography will ultimately be limited by the ability to accurately identify cells in the segmentation step. When tracking cells, the program Celltrack sometimes lost track of cells that were moving too fast into or out of the picture, or moving on top of each other, something which sometimes happened when cells became detached from the bottom.
In less confluent cultures this happened less often, thus giving clearer results. To our present knowledge, a modified watershed algorithm yields the best results, but the algorithm we used could be improved by adding one or several quality checking steps, based on the knowledge of the particular cell line under study, or combining the watershed with other methods such as active contour measurement. Cells were also being lost due to instability of the image field. When computing the phase image, the field of view and the image depth varies with the parameters of the reconstruction. Since we did not use any chemical gradient in this study, the direction of the average movement of all cells was assumed to be zero. Movement was normalized to one cell with a displacement close to the average of the image population, *i.e.*, an “immobile” cell. This method of analysis was evaluated using phase contrast microscopy as well as a pattern in the cell culture vial to study cell movement.

We have shown that the average result for a population does not necessarily properly describe the distribution of motility within the population, as cell motility is usually very individual to the cells.

**Figure 4.** The speed of movement of the individual cells of a single population of L929, L56Br-C1 or MDA-MB-231 cells 1 h after the start of the motility measurements. Images were captured and cells were analyzed at 10-20% confluence. For each population at least 100 cells were analyzed.
It might be more relevant to give the results for the slowest or fastest moving cells in a population as well as the average results. Another factor to consider is that the proportion of motile cells, i.e., the amount of cells that move faster than the image spatial resolution, differs between cell lines. The reduction of motility with increased population confluence was expected [20] and assumed to be a result of contact inhibition. For all three cell lines studied, the fastest 10% of the cells in a population had a larger cell volume than the slowest 10% of the cells. Interestingly, for one cell line, L929, the fastest and slowest 10% had a smaller area than the rest of the cells in the population. For the other cell lines, L56Br-C1 and MDA-MB-231, the fastest and the slowest 10% of the cells had a larger area than the rest of the population. It will be very interesting to see if this is cell line or species dependent. Earlier results indicate that there may be cell line specific differences in cell motility [22].

In our system, the cell motility was much higher during the first hour of the experiment than at later time points. The motility was stable after the first hour. This indicates that there is a change in environment for the cells as they are transferred from the cell culture incubator to a warming plate. Even if the warming plate is supposed to keep the temperature stable at 37°C, it is not as efficient as a cell culture incubator. For future experiments we would recommend an improved system, such as a climate chamber.

In conclusion, a method for marker-free automated microscopic digital holographic tracking, image analysis, segmentation and data extraction was presented. The position of cells in an image was determined subsequently from digital holograms by numerical auto focusing. The applicability of the technique was demonstrated by the quantitative analysis of parameters such as cell confluence, cell area, cell optical thickness and cell volume. These factors can easily be correlated to the cell movement both for entire populations and for single cells or selected parts of a population. We have noted that cells within a population move in very individual patterns with some cells being very mobile and some cells not moving at all.

Figure 5. The average cell movement distance after 5 hours of motility measurements for L929 cells, correlated with confluence. Twenty-two different experiments were performed at different confluences. For each experiment 70-500 cells were analyzed.
Acknowledgement  We thank Helena Cirenajwis at the department of Cell and organism biology, Lund University, Sweden, for supplying us with L56Br6C1 and MDA6MB6231 breast cancer cells.

References


Figure 6. The average distance moved after 5 hours of measurements for ● L929 cells, ▲ L56Br-C1 cells, or ■ MDA-MB-231 cells, correlated to (A) the average cell area, (B) the average cell thickness or (C) the average cell volume of the population. Images were captured and cells were analyzed at 10-20% confluence. The experiments were repeated at least three times with a total of at least 150 analyzed cells.


