A simple method to study cellular migration

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Abstract

We describe here a simple and fast method for the characterisation of cell motion. By projecting on a single plane different positions of the cell a \textit{ribbon} is generated, whose characteristics can be related to the type of motion. The proposed method allows both to determine, very quickly, the motility of a population of cells and to investigate and characterise properties of a single cell’s motion. The methodology presented here can be applied to a large range of cell movement and also adapted and extended to other problems involving biological motion.

\textit{Key words:} computational methods, migration, embryonic CG neurons, bFGF, GDNF

1 Introduction

Cell migration is a basic aspect of the life cycle, both during embryogenesis and in the adult organism. For instance, during embryogenesis, neural crest cells emerge from the dorsal neural tube and disperse along different pathways in the periphery, where their fate is controlled by local environmental signals

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In the central nervous system, the migration of immature neurons from germinal zones to specific positions where axon-target interaction occurs is a critical step in the development of the synaptic circuitry of the brain (Hatten, 2002). Thus, it is not surprising that different disciplines such as biochemistry, biophysics and molecular genetics have all been involved in computer assisted cell motion analysis and in the investigation of signal pathways involved in regulating cellular migration, in order to provide a link between molecules and whole cell, between cells and the whole organism.

The quantitative analysis of cell motility in culture has several important functions: it gives a concise and accurate description of the process and can detect subtle differences in motility due to different experimental conditions; results can be communicated unambiguously to test hypotheses about motility; finally, it may be used to derive a mathematical model and thus to elucidate the underlying mechanisms.

Cell migration can be characterised by different parameters, whose relevance depends on the culture and the biological aspects that are of the interest in the given experiment: among others the speed and the migration angle (Schienbein and Gruler, 1993), velocity components (Distasi et al., 2002), mean-square displacement (Stokes et al., 1991), persistence time and random motility coefficient (Dickinson and Tranquillo, 1993).

In recent years, several methods (Solls and Wessels (1998); Tvarusko et al. (1999); Roy et al. (2002)) have been developed to record the movement of isolated cells through automatic tracking. Unfortunately, measuring cells’ velocity and indeed determining if a cell has moved at all may be a time consuming task as it requires the measurement of the cell position across several frames. The aim of this study is to develop a fast method to visualise and characterise the movement of samples of a cell population. This approach is based on a simple analysis of the cell paths and allows to determine, at a glance, which cells, if any, have moved and to characterise the type of motion of the cell, also capturing the influence of different experimental conditions. These results were obtained by the analysis of the in vitro migration of embryonic chick ciliary ganglion (CG) cells in the presence of different concentrations of basic fibroblast growth factor (bFGF) and glial cell-line derived neurotrophic factor (GDNF), two peptides that stimulate the migration of this cell population.

2 Materials and methods

Cell cultures were obtained as previously described (Distasi et al., 1998). Ganglia, dissected from E7/E8 chick embryos were enzymatically (0.06% trypsin in phosphate buffered saline, PBS, without calcium and magnesium for 5 min
at 37°C) and mechanically dissociated. Cells were resuspended in N2 medium and plated in the middle area of a 40 mm glass coverslip (Bioptechs, USA) for time lapse microscopy. Before plating, the cover glasses were coated with poly-D-lysine (100 μg/ml) alone or with laminin (2 μg/cm²). Human recombinant bFGF (5, 10 or 20 ng/ml, Alomone Labs, Israel) or human recombinant GDNF (50 ng/ml, Alomone Labs, Israel) were added to the culture medium. Starting from two hours after plating, neurons were clearly recognisable from non-neuronal cells by their birifrangent rounded cell bodies and processes: fig. 1 shows a typical image obtained after 24 hours of culture.

Culture purification was obtained adopting the method described in Distasi et al. (2002), which depends on differences in the sedimentation rates of cells in a liquid medium (Davies, 1995). For time-lapse microscopy experiments, at 24 h post-seeding, a micro cover glass was placed in a heated (37°C) live cell chamber system (Bioptechs, USA) on an inverted microscope (Eclipse TE 200, Nikon, Japan), equipped with a CCD video camera (PCO, Germany). If not otherwise specified, all chemicals and drugs were purchased from Sigma Chemical Co. (St Louis, MO, USA).

3 Theory

The motion of cells in vitro, as well as in vivo, is a stochastic process that can be best described by using the Langevin equation (see, for instance, Dunn and Brown (1987); Lauffenburger and Horwitz (1996); Distasi et al. (2002)); even though the behaviour of a single cell cannot be predicted, it is possible to derive the probability density function of the velocity, via the Fokker-Planck equation, and the corresponding momenta (Dunn and Brown (1987); Distasi et al. (2002)).
Fig. 2. Cellular migration visualised via the area swept by the cells: in Fig. 2a the motion results in the relatively large area swept by the cells, whereas in 2b for most cells $A \approx A_0$.

Let cell positions be registered in a sequence of frames $F_1, \ldots, F_n$, corresponding to successive times $j = 1, \ldots, n$. Suppose to arrange these frames in a stack and let $a_i(k)$ be the area occupied by the $i$-th cell in the $k$-th frame. The projection of all frames in a single plane $\tau$ generates, for each cell that has moved, a ribbon, as shown in Fig. 2a, whose area depends obviously on the length of the cell’s trajectory.

More precisely assume, for simplicity, the cells to be circular and denote by $l$ the length of the path of the center of the cell, then

$$l = \int_0^T |\vec{v}| dt = T |\bar{\vec{v}}|$$

(O’Neill, 1966), where $|\vec{v}| = (v_x^2 + v_y^2)^{1/2}$ is the modulus of the velocity vector $\vec{v}$, $T$ is the time interval considered and $|\bar{\vec{v}}|$ is the time average of $|\vec{v}|$.

The maximum area swept by the cell is then

$$A_m = 2rl + \pi r^2,$$
and it is obvious that ribbons areas depend on cell radius and the length $l$ of the cell’s trajectory. Note that the actual area $A$ may be less than $A_m$, as in case of random motion, since on the trajectory $\gamma$ of the cells there may exist points such that their distance, along a line orthogonal to $\gamma$ is less than $2r$, where $r$ is the radius of the neuronal soma.

If a cell does not move, or perform small oscillations around a fixed position, the associated area is $A_0 \approx \pi r^2$; in general, $A >> A_0$ making it very easy to detect, by visual inspection or by some automatic method, which cells have moved.

A simple quantitative measure of the motion of a population is the average value $\bar{A}$ of the area covered by each cell,

$$\bar{A} = \frac{1}{N} \sum_{i=1}^{N} A_i.$$  

(3)

We are dealing with homogeneous populations, then differences in size of single cells are smoothed out by averaging operation. Fig. 2a and 2b show the results obtained in two prototypical cases: in the first one the motion of some cells is apparent, whereas in the second one the majority of cells has not moved. Here the averages $\bar{A}$ are 344 $\mu m^2$ and 118 $\mu m^2$, respectively, a ratio of almost 3 to 1. Other examples are shown in Fig. 3. In these figures the number of cells was estimated by computing the ratio between the total area covered by the neurons and the mean area of a single neuron (a check was carried out by visually counting the cells).

Beside providing a way to estimate the motility of populations this method also allows to characterise motion of a single cell in a very simple way. As can be seen from Figs. 4a and 4b trajectories of cells fall in two classes: some of them turn on itself so that the net distance $d$ between the starting and final points is very small, others are more linear and $d$ is not very different from $l$. In the following, paths of the first type will be called $R$-trajectories, and those of the second class $D$-trajectories. It is straightforward to show that these two classes are related to the statistics of the cell velocity components $v_x$, $v_y$ (Distasi et al., 2002). If both averages $\bar{v}_x$, and $\bar{v}_y$ are not significantly different from zero then $d \approx 0$, and a $R$-trajectory arises, whereas if at least one of $\bar{v}_x$, $\bar{v}_y$ is significantly different from zero $d$ tends to approximate $l$ and the corresponding trajectory will be of type $D$. These types of motion and trajectories can result from different classes of forces acting on the cells: cells undergoing an $R$ motion are affected solely by random forces, whereas $D$-motion is due also to the action of deterministic forces (for details see Distasi et al. (2002)).

The two basic types of ribbons and associated trajectories shown in Fig. 4 can
be given a quantitative characterisation in a variety of ways, for instance via the ratio \( J = d/l \); however, parameters exist that can be computed directly from the *ribbons* namely the circularity \( C \) and the Feret’s diameter \( F_d \). Let \( A \) be the area of a *ribbon*, circularity \( C \) is defined as \( C = 4\pi(A/P^2) \), where \( P \) is the perimeter: \( C = 1 \) characterises a perfect circle and values close to 0 correspond to elongated polygons. The Feret’s diameter, also known as the caliper length, is defined as the longest distance between any two points along the boundary of \( A \). Thus, if the trajectory turns into itself in a random way we should expect \( C \approx 1 \) and \( F_d \) to be relatively small, conversely for an almost
Fig. 4. Comparing different types of trajectories: 4a the motion of the cell is purely random, whereas 4b shows the area corresponding to a $D$ motion.

straight paths $C \approx 0$ while $F_d$ attains a comparatively large value. Note that, by definition $C$ is insensitive to cell’s size, on the other hand the $F_d$ is in any case much larger that the cell’s diameter.

4 Computational issues

In our experiments images were acquired every 20 s (acquisition board and software LabVIEW, National Instruments, USA). Experiments were carried out over a period of about 10h. Images were analysed by using the GIMP (GNU Image Manipulation Program) and ImageJ\footnote{ImageJ can be downloaded from ftp://rsbweb.nih.gov/pub/image-j/, in different versions for Windows, MacOS X and Linux. ImageJ was designed with an open architecture that provides extensibility via Java plug-ins. Custom acquisition, analysis and processing plug-ins can be developed using ImageJ’s built in editor and Java compiler.} (Rasband, 2002) , an image processing and analysis software developed in Java(TM), on a Debian/GNU Linux machine. Details of the implementation and the software can be requested at the email address paolo.ariano@unito.it

Several methods are available to segment the images and extract cells from the background. In the present case, however, since cell images have much higher grey level values than the background a simple threshold will suffice.

Fig. 5 presents a typical histogram of grey level values ($gl$); it shows a peak around $gl = 100$, corresponding to the background, and another at $gl = 255$, due to the cells bodies. It is clear that any threshold value larger than $gl = 200$ separates cells from the background. This way we have obtained binary images, where the bodies of the neurons are white, whereas the background, including glial cells, is black.

In our experiments the first step was to check if the number of cells in the considered field is constant. In the case of a purely random process this is usually the case, at least approximately, whereas if some chemoattractant is present
the number of cells may change with time, increasing if the chemoattractant is inside the field or decreasing if it is outside. Checks on the number of neuron have been carried out by inserting in ImageJ a subroutine that counts the total number of white pixels; after the application of the threshold this is just the area covered by the neurons, and then it must be constant in time, that is in any frame.

Images showing ribbons were obtained by building a stack of images at different time and projecting it onto a single plane. Note that ImageJ can display a stack of related images in a single window, process them using a single command, open a folder of images as a stack, save stacks as multi-image TIFF files, and project the images of the stack in a single plane; both maximum and average intensity projections are supported. Areas of cells and ribbons depend on the set-up parameters, however what is of interest is the ratio of these areas rather than their absolute values, so that the method is applicable for different set-ups.

Finally also Circularity and Feret’s Diameter can be calculated directly by ImageJ by means of a single command.

5 Results

We have carried out a series of experiments to check that the method can indeed detect different patterns of cell motility. At first we compared the extent of neuronal migration in the presence of two different concentrations of bFGF,
Table 1
Circularity and Feret’s diameter calculated from trajectories in Fig. 4.

<table>
<thead>
<tr>
<th>Circ.</th>
<th>Feret</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>0.24</td>
</tr>
<tr>
<td>R</td>
<td>0.77</td>
</tr>
</tbody>
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a peptide that plays several functions in the nervous system (Ford-Perris et al., 2001). In particular in developing chick CG cells in vitro, bFGF strongly stimulates the migration and the aggregation of neurons and glia (Distasi et al., 1998). The extent of migration has been evaluated by mean of the average value of the area covered by the neurons (see Eq. 3); for a concentration of 20 ng/ml of bFGF $\bar{A} = 678 \mu m^2$, whereas for 10 ng/ml $\bar{A} = 144 \mu m^2$, a ratio almost five to one (see Fig. 3a, b). Next, we considered the effect of GDNF (50 ng/ml), a chick CG target derived factor that promotes survival on many types of peripheral and central neurons (Hashino et al. (2001); Araiksenen and Saarma (2002)). Both bFGF and GDNF are able to stimulate the aggregation in vitro of chick CG neurons and glial cells and a qualitative analysis has shown that bFGF increases the migratory activity more than GDNF (Zamburlin et al., 2003). These data have been quantitatively confirmed: when 50 ng/ml GDNF was present $\bar{A}_1 = 251 \mu m^2$, a result much lower than the one obtained with 20 ng/ml of bFGF ($\bar{A}_2 = 678 \mu m^2$, $\bar{A}_2/\bar{A}_1 > 2.5$ see Fig. 3a, c ).

Finally, it has been argued that Circularity and Feret’s diameter can characterise two fundamentally different types of motion, as shown in Fig. 4: R-motions are expected to show high $C$ and low $F_d$, the converse should be true for D-motions. An example is shown in Table 1 where $C$ e $F_d$ has been computed for the cells of Fig. 4: the results demonstrate that indeed the characterisation provided by these parameters is consistent with the statistics of velocity components and hence that $C$ and $F_d$ values discriminate different types of cell behaviour. Computation of $C$ and $F_d$ is much simpler to carry out than the analysis of velocity components, even though, obviously, the latter provides more information about the nature of the motion.

6 Conclusions

In this study we have developed a new method that enables rapid analysis of cell movement. As shown in the results section the method allows not only to determine which cell in a given population has moved, but also to perform a preliminary analysis of the types of forces acting on the cells and the extent of the migration in the presence of different environmental conditions. The classification of cells motion obtained here is consistent with results obtained by approaches computationally more expensive, involving cells tracking (Distasi
et al., 2002).

The present method cannot deal with complex situations, such as overlapping of ribbons, or other occlusions; it must be observed, however, that occlusion is a difficult problem for any motion understanding system.

Several softwares are available on the market which are able to track cells and provide up to 100 parameters of motility and dynamic morphology for each cell. However, they are expensive and require large hardware to acquire, store and process images. The aim of this study, on the other hand, was to develop a simple method to obtain qualitative and quantitative information on cell motion in a fast and simple way, computationally very inexpensive.

Obviously the approach presented here can not, and is not intended to, replace more complex approaches/softwares, but it can be useful for performing a preliminary analysis and to tune the parameters involved in complex softwares that often produce redundant information not relevant to the problem under study.

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References


