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Investigation of Cell Dynamics *in vitro* by Time Lapse Microscopy and Image Analysis

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Pharmacological research is continuously working on the development of new drugs. This research typically starts from the formulation of new molecules that are first investigated at the cell scale, finally is completed with clinical trials. Investigation on the cell scale requires simple, reproducible and reliable assays, able to simulate physiological conditions in the lab.
A wide range of biological processes, such as angiogenesis, inflammation, tissue regeneration, tumour growth and invasion, are strongly linked to cell proliferation and migration mechanisms that govern the dynamic evolution of both individual cells and cell aggregates.
In this work we present an experimental methodology for the quantitative investigation of cell dynamics *in vitro* by live imaging of biological soft matter. Cell motility is observed by means of a Time Lapse Microscopy workstation, consisting of a motorized video-microscope equipped with an incubating system, and quantified by image analysis techniques. We report some preliminary experimental results relative to the migration of a tumour cell line both in random condition and in presence of an external stimulus, such as a chemical concentration gradient. The ultimate goal of this research is the development of a standard assay to be used as a test for drug efficiency, suitable for routine application in the pharmaceutical research.

1. Introduction

The pharmacological industry is strongly addressed to discovering and testing of novel therapeutic drugs for the treatment of a wide range of diseases, including cancer, inflammations and cardio-vascular dysfunctions. In particular, the identification of novel chemotherapeutic molecules is a topic of growing interest, because of the limitations of the therapy approaches to the treatment of cancer, due to tumour invasion and metastasis formation.
The research process of novel drugs is complex, time-consuming, and expensive. A wide variety of *in vitro* assays are used to identify novel therapeutic molecules and assesses their efficiency on the industrial scale. Among these, the Boyden chamber assay (Boyden, 1962) is widely used to investigate cell motility and invasion capacity, due to its simplicity. However, it presents a number of limitations; it does not allow cell dynamics to be monitored as a function of time and does not provide well defined concentration gradients of the drug under evaluation.
The detection of novel drugs and the evaluation of their activity require the development of a standard assay aimed at the investigation of single cells and cell tissue dynamics in response to drug treatment, while mimicking physiological condition on the lab scale. An ideal assay should be economic, relatively simple to set up with significant reproducibility and reliability, and useful for high-throughput screening.

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A large number of physiological and pathological processes, including embryonic development, immune response, inflammation and tumour metastasis, are intimately related to the dynamic evolution of individual cells and cell clusters (Friedl and Gilmour, 2009). Cell migration, proliferation and aggregation mechanisms, driven by mechanical and chemotactic cues (Roussos et al., 2011), play also key roles in the growth of healthy as well as pathological tissues.
The above mentioned mechanisms of cell dynamic evolution can be described by using mathematical models based on transport phenomena concepts (Ottino, 2011). It is possible to describe cell motility in terms of a motility coefficient, analogue of the Fickian diffusion coefficient, while cell proliferation can be described by models of logistic growth (Tremel et al., 2009). The fusion of two contiguous cell aggregates may be described in terms of an effective interfacial tension (Pommella et al., 2013) that promotes the formation of clusters with minimum external surface. An interesting approach of this phenomenon can be hence based on the analogy with the case of drops of fluid surrounded by an immiscible matrix (Preziosi et al., 2013), that deform, break-up (Caserta et al., 2013a), retract to the spherical unperturbed shape, coalesce, or form complex structures (Caserta and Guido, 2012).
A detailed analysis of the mechanisms leading cell dynamics requires a rigorous approach, based on the measurement of quantitative cell movement indices. Cell dynamics can be efficiently investigated experimentally *in vitro* by using live cell imaging by Time Lapse Microscopy (TLM), that allows the direct visualization of biological systems during their dynamic evolution (Terryn et al., 2009). This microscopy technique is based on automated sample repositioning by motorized x-y stage and focus control and iterative image acquisition of selected fields of view while controlling the environmental parameter to ensure cell viability throughout the experiment, which can last up to a few weeks. The application of image analysis techniques allows the reconstruction of cell trajectories and the calculation of the relevant cell migration parameters (Buonomo et al., 2012), or the quantification of the growth dynamic of tissues (Silano et al., 2012).
TLM is coupled to a large variety of *in vitro* assays, ranging from single cell random motility assays to chemotaxis assays, that allow a quantitative characterization of cell dynamics (Kramer et al., 2013).
In single cell random motility assays, the cells are plated at low density, in order to reconstruct cell trajectories in 2-D or 3-D substrata. The trajectories are further analyzed according to mathematical model, such as the persistent random walk model (Dickinson and Tranquillo, 1993), in order to measure motility parameters, i.e. cell velocity, the persistence time between significant changes in the direction of motion, and the cell motility coefficient, that is an analogous of the random walk diffusivity.
Chemotaxis assays allow to quantitatively analyze the directional cell response to chemical gradients. The investigation of chemotaxis *in vitro* presents several challenging experimental difficulties, mainly due to the problem of creating a stable gradient on a time scale long enough to elicit a significant cell migration response (Caserta et al., 2013b). Chemotactic movement of the cells can be described in terms of a directionality index, defined as the ratio between the net movement in the direction of the gradient and the total curvilinear length of cell trajectories (Vasaturo et al., 2012).
In this work, we propose an experimental methodology for the quantitative investigation of cell dynamics *in vitro* by live imaging of biological soft matter. Cell motility was observed by means of time lapse imaging and quantified by image analysis techniques. We report some preliminary experimental results relative to a case study aimed at the investigation of tumour invasion. In particular, we performed 2D single cell migration assays in order to quantitatively investigate the dynamic behavior of two populations of tumour cells, i.e. HT1080 NG2- and HT1080 NG2+ fibroblasts. The latter are characterized by high expression of the NG2 transmembrane proteoglycan. It is thought to be involved in tumour progression (Cattaruzza et al., 2013) because it accentuates growth responses, mediates the tumour cell-host microenvironment interaction and promotes neoangiogenesis (Benassi et al., 2009).
In order to investigate the chemotactic response of the cells to several molecules, we applied a novel chemotaxis assay in 3-D collagen gels based on a direct-viewing chamber (Caserta et al., 2013b, Vasaturo et al., 2012) that is reusable, and can be coupled to a Time Lapse Microscopy and image analysis workstation. In the chamber a chemoattractant concentration gradient in the collagen gel sample seeded with cells is generated by diffusion through a porous membrane. We analyzed the migration of HT1080 tumour fibroblasts under a concentration gradient of FGF2 growth factor, that is known to be implicated in the progression of human cancer (Berger et al., 1999).

2. Materials and methods

2.1 Cell culture

HT1080 is a cell line of fibrosarcoma from connective tissue. This cell line presents good cell motility and a significant morphological polarization. HT1080 fibroblasts and two subpopulations of this cell line (HT1080

NG2+ and NG2-) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % (v/v) Fetal Bovine Serum (FBS), sodium pyruvate 1 % and antibiotics (50 units/mL penicillin and 50 µg/mL streptomycin) and maintained in a humidified incubator at 37 °C under an atmosphere of 5 % CO_2 in air.

2.2 Experimental methods

In 2D random motility experiments, HT1080 NG2+ and NG2- fibroblasts were plated on an uncoated twelve-well culture dish at a density of $9x10^4$ cells per well and allowed to attach overnight, incubating under standard conditions at 37 °C in a 5 % CO_2/Air atmosphere before starting the experiments.

Chemotaxis assays were performed by using a chamber and a Time Lapse workstation designed to maintain both cell viability and good optical quality over a time scale of at least 24 h. The chamber (Caserta et al., 2013b, Vasaturo et al., 2012) consists of a single aluminium block glued on top of a microscope slide by using a silicone adhesive. A porous membrane (0.22 µm pores), sandwiched between two rectangular metal frames, separates two compartments, one for the cell seeded collagen gel (sample well), and the other as a reservoir of the chemoattractant solution (chemoattractant reservoir). In Figure 1.A, a 3D rendering shows the chamber in an assembled view, where the membrane supporting frames are housed in place. Collagen gel was prepared with the following composition (volume basis): DMEM medium, 0.1 M NaOH (5 %), 10x MEM medium (5 %), FBS (0.5 %), antibiotics (50 units/mL penicillin and 50 µg/mL streptomycin) (1 %) and collagen solution (2 mg/mL). All components were kept on ice during the preparation, except for the cell suspension that was added at the end. The solution was placed in the sample well of the chamber. The chamber was then incubated at 37 °C and 5 % CO_2 for 20 min to induce collagen polymerization. During the experiment the chemoattractant, loaded in the reservoir, diffuses through the membrane, thereby generates a concentration gradient in the cell seeded collagen gel. The chemoattractant concentration profile in the collagen gel can be described according to the model of Fickian diffusion in a semi-infinite slab (Caserta et al., 2013b):

$$C(x,t) = \frac{C_0}{2}\left[1 - erf\left(\frac{y}{\sqrt{4Dt}}\right)\right] \quad (1)$$

where $C(x,t)$ is the chemoattractant concentration as function of the space x and time t, C_0 is the initial concentration in the chemoattractant reservoir, D is the diffusion coefficient of the molecule in the gel.

2.3 Time Lapse Microscopy workstation

Time Lapse Microscopy experiments were performed using an automated workstation based on an inverted optical microscope with a long working distance 10x objective in phase contrast. A scheme of the workstation is reported in Figure 1. The microscope, placed on an anti-vibrating table, is equipped with motorized stage and focus, that allow to automatically position the field of view within the sample under observation. In order to mimic environmental conditions, the microscope is enclosed in a homemade incubator that keeps the sample temperature at 37 ± 0.1 °C in a saturate moisture atmosphere with 5 % CO_2. Images are acquired using a high-resolution high-sensitivity monochromatic CCD video camera. The whole workstation is driven by homemade control software in Labview. Images were stored on hard drive for off line analysis.

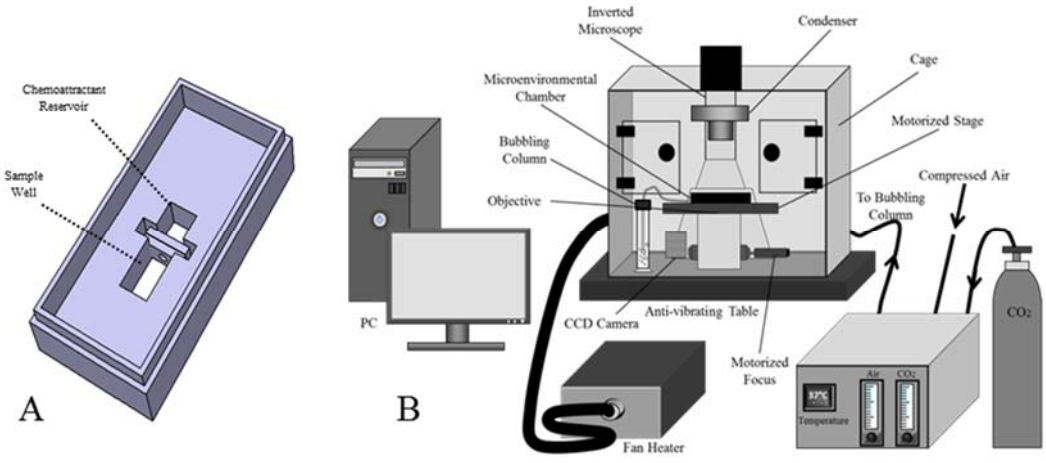

Figure 1: View rendering of the chemotaxis chamber (A) and scheme of the Time Lapse Microscopy workstation (B).

2.4 Image analysis

Image analysis of random motility assays and chemotaxis experiments was performed using a semi-automated Cell Tracking software. For each time step, all the cells were individually followed to determine cell contour and the coordinates of the center of mass. The trajectory of each cell was reconstructed for the whole experiment starting from the center of mass coordinates. Furthermore, average motility parameters of the cell population were calculated as a function of time using a Matlab script.

3. Results and discussion

We performed 2D single cell migration assays, in order to quantitatively investigate the dynamic behavior of two populations of cancer cells, i.e. HT1080 NG2- and HT1080 NG2+ fibroblasts.

A detailed analysis of the motility of the two fibroblast populations on a planar surface is reported in the following. In Figure 2A and B we report the trajectories described by HT1080 NG2- and HT1080 NG2+ fibroblasts respectively; cell paths are plotted starting from the same initial position. In both fibroblast populations, the trajectories showed a random orientation being uniformly distributed on the XY plane (i.e., no preferential direction in cell motion can be distinguished). However, more extended trajectories were detected in NG2+ compared to NG2- fibroblasts.

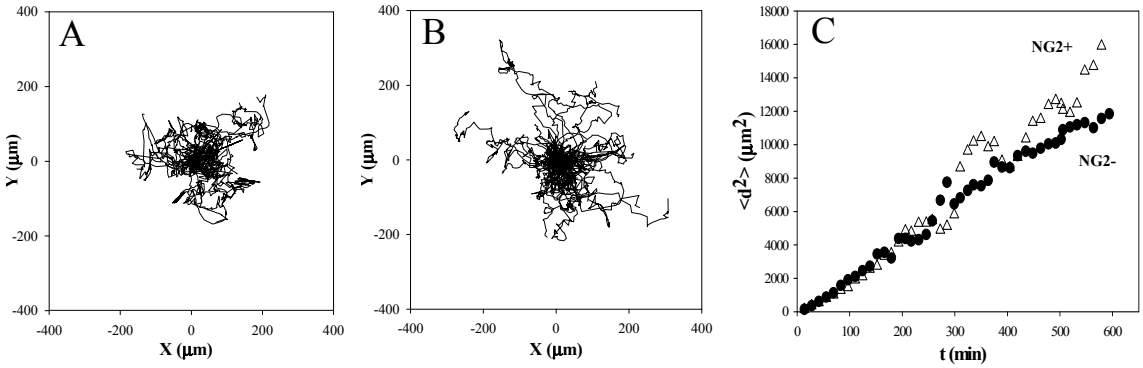

Figure 2. Fibroblast trajectory analysis and mean square displacements. A direct analysis of cell trajectories is used to characterize the motion of NG2- (A) and NG2+ fibroblasts (B). To quantitatively assess cell movement, the mean square displacements are calculated (C).

Cell motility was quantified by measuring quantitative motility indices, according to the persistent random walk theory (Dickinson and Tranquillo, 1993), where it is assumed that cell motion is characterized by a diffusion coefficient (also referred to as the random motility coefficient) μ (μm²/min) and a persistence time P (min), that is, the characteristic time in which cell movement persists in the same direction. The value of μ is related both to the average speed of the cells and to the persistence time. According to the theory, the mean square displacements are given by the equation

$$\langle d^2(t)\rangle = 4\mu\left[t - P\left(1 - e^{-t/P}\right)\right] \tag{2}$$

where $\langle d^2(t)\rangle$ (μm²) is the mean squared displacement of the tracked cell sample at time t. The trend predicted by Eq(2) is linear for $t \gg P$, with a slope proportional to the diffusion coefficient (i.e., $\langle d^2(t)\rangle \approx 4\mu t$). The squared displacement was measured for every interval by calculating the Euclidean distance between the two positions occupied by the cell at the beginning and at the end of the interval. The mean squared displacements $\langle d^2(t)\rangle$ (μm²) were then calculated as an average over the number of measurements done for each cell over the entire trajectory, and then over the entire cell population. In Figure 2C we report the mean squared displacements as a function of time, for HT1080 NG2- and NG2+ fibroblasts. Eq(2) was fit to the experimental data of mean squared displacements as a function of time, with μ and P as the only adjustable parameters. The velocity of the cells V was also calculated as the ratio between the curvilinear trajectory described and the elapsed time, averaged over the entire cell population. The statistical significance of the results was verified by repeating the analysis on another identical cell population, for each sample.

In Table 1 we report the estimate of the motility parameters (μ, P and V) for the two fibroblast populations; the standard deviation is reported as uncertainty.

Table 1: Motility parameters for HT1080 NG2+ and HT1080 NG2- fibroblasts.

Cell sample	μ (μm²/min)	P (min)	V (μm/min)
HT1080 NG2-	5,50±0,15	32,19±0,10	0,59±0,01
HT1080 NG2+	7,11±0,31	39,58±1,85	0,61±0,03

HT1080 NG2+ fibroblasts show higher motility compared to NG2- fibroblasts, as evident by the higher value of all the parameters examined, especially the random diffusion coefficient μ and the persistence time P.Our preliminary results support the hypothesis that the NG2 proteoglycan is involved in the regulation of cell motility. Further experimental investigation is in progress to confirm this result.
We also analyzed the chemotactic response of HT1080 cancer fibroblasts under a concentration gradient of FGF2. This growth factor is known to be implicated in the progression of human cancer.
The directionality of cell movement during chemotaxis assays was quantified by defining a directionality index I, that is the ratio between the net movement in the direction of the gradient and the total curvilinear length of the cell trajectory. It ranges from +1 (trajectory fully oriented towards the source of chemoattractant) to −1 (negative chemotaxis). I = 0 corresponds to a random motion where no preferential direction is observed.
In Figure 3A we report the quantitative measure of the y component of the chemotaxis index (Iy), i.e., the ratio between the net displacement in the direction (y) of the gradient and the total curvilinear trajectory of the cells, as a function of time. Iy fluctuates around 0, meaning that the fibroblasts seem to move in a random fashion. The average cell velocity on the other hand shows a progressive increases in the first 7 hours, as shown in Figure 3B. This result suggests a chemokinetic, rather than chemotactic, effect of FGF2 growth factor on HT1080 fibroblasts.

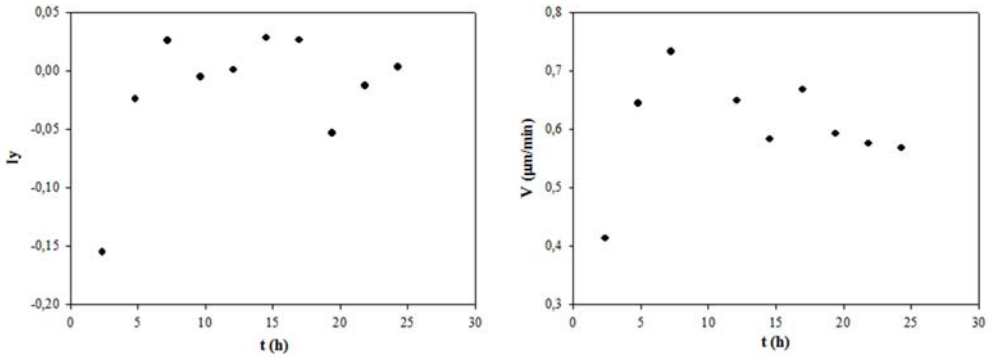

Figure 3. Analysis of motility for HT1080 fibroblasts under FGF2 chemotactic gradient: the y component of the chemotactic index (A) and average cell velocity modulus (B) as a function of time.

5. Conclusions

The development of physiologically relevant *in vitro* assays to identify novel therapeutic molecules and test drug efficiency is a topic of growing interest in the pharmacological industry, mostly due to the wide application in the treatment of cancer.
Due to the complexity of the cell response, a detailed quantitative assay requires an interdisciplinary approach based on chemical engineering core disciplines combined with biological and biomedical sciences (Ottino, 2011). A rigorous investigation, based on the application of transport phenomena concepts, is essential to measure cell movement indices that describe the dynamic response of cells to drug treatments.
In this work we present an experimental methodology to investigate the dynamics of biological soft matter in a quantitative way, while mimicking physiological condition on the lab scale. In particular, we used Time Lapse Microscopy in order to analyse the motility of a tumour cell line both in random condition and in presence of an external stimulus, such as a chemical concentration gradient. We applied a novel methodology for the experimental investigation of drug efficiency *in vitro* by time lapse live cell imaging of cell movement under a controlled gradient of a soluble molecule. In this assay, a concentration gradient in a collagen gel sample seeded with cells was generated by diffusion through a porous membrane. Preliminary results are reported to validate the technique.

The aim of this work is the development of a standard assay to be used as a test for drug efficiency. The technique proposed provides highly reproducible results (Caserta et al., 2013b, Vasaturo et al., 2012) and is promising for routine application in the pharmaceutical industry.

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