# MONITORING THE PARAMETERS OF CELLS MIGRATING IN PSEUDO-3D EXTRACELLULAR MATRIX

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**Abstract**: Cell migration plays an essential role in a number of biological processes, such as embryogenesis, the immune response, wound healing and inflammation. Most research regarding cell migration is based on experiments using two-dimensional (2D) cell cultures, and the detailed molecular and biophysical mechanisms of these processes are already well known. However, much less is known about cell behaviour in the three-dimensional (3D) environment of living tissues. Pseudo-three-dimensional (pseudo-3D) cell cultures bridge the gap between 2D and 3D geometries and combine their advantages. This review presents the quantitative evaluation of human mesenchymal stem cells (hMSCs) migration parameters in pseudo-3D artificial extracellular matrix (ECM) of various compositions.

**Keywords**: hMSCs, Pseudo-3D, Extracellular matrix, Collagen, Confocal microscopy.

## 1 INTRODUCTION

Various methods are currently used to study cell migration. In vitro model systems based on cell culturing are most often used. The experiments are most often performed on 2D cell cultures, where the cells are in a form of a monolayer on different surfaces, most often on a plastic or a glass [1, 2]. Such experiments include, for example, a scratch assay, where a scratch is made into a cell monolayer and the cell migration towards the centre of the scratch is monitored. The advantages of 2D cell cultures include simple and economical implementation; shorter time required for cell culture formation in the range of minutes to hours; and high speed of proliferation. However, these 2D systems are not surrounded by their natural microenvironment, and their responses to various chemicals or other stimuli may differ significantly from those found in a living organism. Cells lack components from the ECM, including proteins (especially collagen (CG)), glycosaminoglycans and glycoproteins (laminin, fibronectin (FN)).

However, differences between cell behaviour in culture and *in vivo* have gradually led to the transition to 3D models that better represent the microenvironment of living tissues [3]. Here, the shape of cells, the interactions between them and the environment, better mimic the natural environment. Such models contain not only proliferating cells, but also resting cells and apoptotic, hypoxic or necrotic cells. These models have the ability to better reflect normal differentiation, cell behaviour, and intercellular interactions. Cells cultured in a 3D environment show adhesion to the intercellular matrix with their entire surface. This environment not only physically supports cells, but also provides information that can affect cell differentiation in some way.

These important features make 3D cell cultures more physiologically relevant and predictive than 2D cultures. However, these 3D systems have their limitations, especially in microscopic imaging and data evaluation. Imaging may become difficult depending on the material transparency and scaffold size. The creation of pseudo-3D cell cultures allows to combine the ease of 2D systems while overcoming some limitations of 3D systems. Therefore, a pseudo-3D culture was defined as a culture where cells are embedded in the ECM but in contact with coverslip. [4]

A network of ECM molecules are basic building blocks of such 3D and pseudo-3D models, which is often in the form of a gel. The construction material must have a specific structure and spatial arrangement of molecules to ensure cell growth and migration. Sufficient porosity should be provided for uniform cell proliferation, migration and growth, both in the scaffold space and over time. An important parameter of the material is its biocompatibility. This is compatibility with living tissue, with the material not being toxic, harmful, or physiologically reactive. The cells must be able to adhere to the material, migrate to the surface and grow and proliferate in the scaffold. Such material must also be non-immunogenic, so as not to cause any adverse reactions (inflammation, cytotoxicity, mutagenicity, etc.) [5]. The materials used to form these support matrices are generally of two types. In the first case, these are biological polymers such as fibrin, collagen, hydrogel, chitosan. In the second case, synthetic polymers such as polylactic acid (PLA) or similar materials such as polyglycolic acid (PGA) and polycaprolactone (PCL) can be used.

Collagen, together with elastin and hyaluronic acid are the most important components of the ECM that are predominantly produced by fibroblasts. The collagen gel is bioactive and has cell adhesion promoting properties [6]. Another principal part of the ECM is fibronectin. It is a the most abundant molecule of the extracellular matrix and it belongs to the most studied ECM components. FN increases cell adhesion and proliferation; affects cell migration pathways *in vivo* and in culture; and specifically stimulates cell proliferation. FN also plays a role in cell morphology, cytoskeletal organisation, haemostasis, and wound healing. The study [7] showed that the addition of FN to cells cultured in a collagen gel led to an increase in the number of cells and their motility.

The goal of our experiment was to focus on the study of cell migration in pseudo-3D collagen gel, simulating an ECM environment in order to determine the influence of the gel environment on the parameters of cell migration. In addition, this experiment was extended to study the effect of FN on cell migration. The hMSC migration was monitored using a confocal microscope. The hMSC cell line that is considered the gold standard for clinical research was chosen for this experiment.

## 2 MATERIALS AND METHODS

### 2.1 DESCRIPTION OF THE EXPERIMENT

The ECM was prepared from rat tail collagen type I (Ibidi GmbH) and FN (Sigma-Aldrich s.r.o.). In this paper hMSCs isolated from a human adipose tissue obtained by abdominal liposuction were used. Cells were cultured in a low glucose Dulbecco's Modified Eagle Medium (DMEM) containing 5% foetal bovine serum (FBS) and 1% of penicillin-streptomycin (100 $U \cdot ml^{-1}$:100 $\mu g \cdot ml^{-1}$) at 37°C and 5% $CO_2$. All chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

In our experiment, cells were seeded in one well of a 24-well plate (Sigma-Aldrich). When cell confluency reached 80-90%, then cells were stained with CellTracker™ Green CMFDA Dye (CMFDA; Invitrogen™) in a final concentration of 1 µM in serum-free medium for 15 min. Cells were gently washed by phosphate-buffered saline (PBS) before and after staining. Then, 750 µl of rat tail collagen type I (ibidi GmbH) in a final concentration of 1 $mg \cdot ml^{-1}$ were prepared in a microtube and placed on ice. Then 200 µl of prepared collagen gel was placed into the second microtube and mixed with 80 µl of FN in a concentration of 10 $\mu g \cdot ml^{-1}$. In the third microtube 200 µl of collagen gel were mixed with 80 µl of FN in a concentration of 50 $\mu g \cdot ml^{-1}$.

To study the hMSC migration in ECM, 8-well chambered cover glasses (#1.5H, Cellvis) were chosen in order to observe all of the studied ECM types simultaneously. One of the wells was filled with 500 µl of growth medium (control). The next three wells were filled with 250 µl of each prepared gel (CG and CG/FN). Then for gel polymerization, the cover glass was placed into a humidified incubator (37°C, 5% $CO_2$) for one hour. At this moment, the previously stained cells were passaged using accutase (PAA Laboratories GmbH). When the gel polymerization was complete, then 125 µl of growth medium was added to the surface of the gels. Then cells were seeded in the well with growth medium and the three wells with the gels at a density of $4 \cdot 10^3$ $cells \cdot cm^{-2}$. After that, the cover glass was placed into an incubator for four hours. During this

time cells were penetrated into the gels and adhered on the bottom of the cover glass. After this, the cover glass was placed into the confocal microscope incubator chamber (The Stage Top Chamber, OKOLAB). Five hours after cell seeding, the image acquisition was started. Thus, the atmosphere of 5% $CO_2$ and 37°C temperature was maintained during the entire length of the experiment.

### 2.2 DATA ACQUISITION

The data were acquired using a Leica TCS SP8X confocal microscope equipped with White Light Laser (WLL). The samples in the micro-chamber were observed using a lens with 10X magnification. Cells were labelled with CellTracker™ Green CMFDA fluorescent dye, which can be retained in living cells through several generations (72 hours). Excitation wavelength was set to 490 nm and an emission range of 500–540 nm, corresponding to the dye spectral properties. To follow the hMSC migration, time-lapse data were acquired; the stacks of 67 images were obtained every 15 min over a period of 16 hours and 30 min. The volume included in an image stack is of the size 1.16 × 1.16 mm with a spatial resolution of 512 × 512 pixels for each image.

### 2.3 DATA ANALYSIS

The aim of processing the image sequences obtained from the experiments is to determine the necessary migration parameters, such as the total distance travelled, the net distance and the speed of cell motility. Based on these parameters, a comparison of the four tested groups and evaluation of the results of the experiments was performed. An algorithm in the MATLAB software environment (version R2020a) was designed for the semiautomatic analysis of confocal image stacks. The base of the algorithm (Figure 1) is the cycle of the opening microscopic images and marking the positions of selected cells, until all images from the entire set are processed. The given cell density and time interval of image acquisition were sufficient to ensure that manual cell marking was objective and that there were no errors in determining the position of a selected cell throughout the image sequence. Cells were cultured between a cover glass substratum and soft gel layer, and the experiments did not indicate cell migration into the gel layer, so, the cells were always in a single focal plane. The obtained groups of coordinates are further used for numerical calculations and representations of the obtained traces (directional roses, Figure 3).

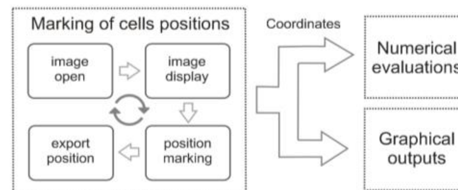


**Figure 1:** Scheme of the proposed algorithm.

In the first step, the cells of interest were manually marked in each first image of the sequence. Then the cells were tracked using processing of all images in the sequence. Due to the different image sizes, the data were normalized by converting pixels to micrometres (1 px = 2.275 µm). The recalculated routes were used as input data for plotting in the directional rose. The total distance (also as Accumulated distance) travelled is then also calculated from the obtained tracks:

$$d_{total} = \sum_{i=1}^{n-1} \sqrt{(x_i - x_{i+1})^2 + (y_i - y_{i+1})^2} \tag{1}$$

where $x_i$ and $y_i$ are the coordinates of the cell in the i-th image, $x_{i+1}$ and $y_{i+1}$ denote the coordinates of the same cell in the (i+1)-th image, n denotes the total number of images in the sequence. The net distance (from the start to the end position, also as Euclidean distance) is calculated as

$$d_{net} = \sum_{i=1}^{n-1} \sqrt{(x_{end} - x_{ini})^2 + (y_{end} - y_{ini})^2} \tag{2}$$

Knowledge of the time interval of image obtaining allows to calculate the velocity of individual cells and also the velocity of all monitored cells in a given experiment.

## 3 RESULTS AND DISCUSSION

The presented algorithm was used to evaluate the migration of hMSCs in a pseudo-3D environment. Three independent experiments were performed with cells in collagen gel with a collagen concentration of 1 mg·ml$^{-1}$. The principle of each experiment consisted of long-term scanning in four wells, which formed four experimental groups. After applying the proposed algorithm to microscopic image stacks, the migration parameters were obtained (Figure 2).

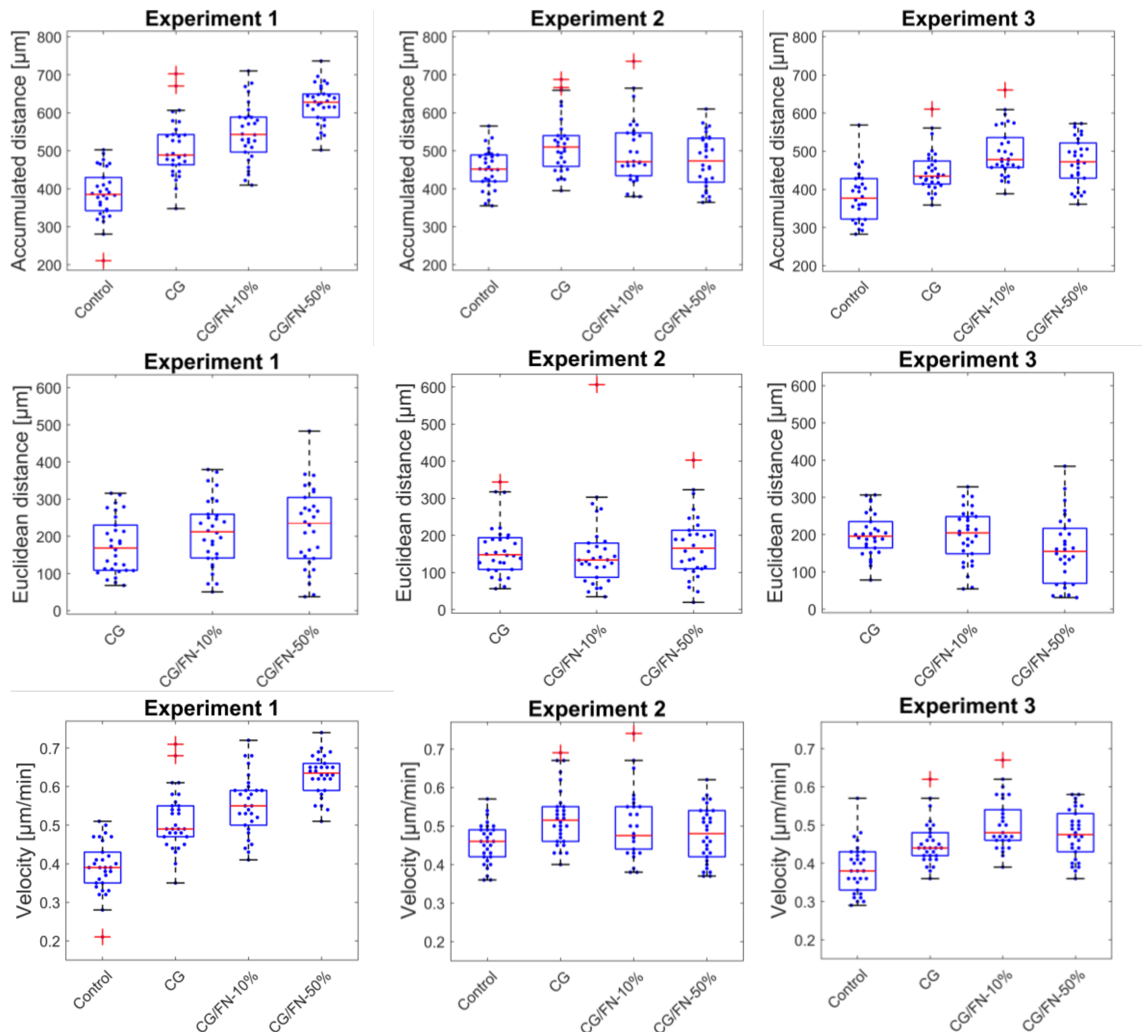


**Figure 2:** Migration parameters obtained during the experiments.

The numerical results were further used for analysis using statistical tests. The aim was to verify whether the average value of these parameters was approximately the same in all groups. A one-way ANOVA (MATLAB, anova1) was used for evaluation. A test on the identity (homogeneity) of the variances (F-test in MATLAB, vartestn) and Shapiro-Wilk test were used at first to verify the assumption of a normal distribution and agreement of the variance of the data in the groups. The results show that the difference between the individual groups is insignificant (p-values > 0.05).

The control group included cells that were cultured only in the presence of medium. Cells in the other groups were cultured in a collagen gel, that was a higher density environment. The result showed that there was a significant difference between the groups in all experiments – cells cultured in collagen gel move at a higher velocity (Figure 2).

The aim of further statistical tests was to determine whether the addition of FN affects the direction of cell migration and the speed of cell motility (Figure 3). The same statistical tests were used as before. The control group contained cells that were cultured in collagen gel, and other groups included collagen with FN at 10% concentration and 50% concentration. The effect of FN on cell migration speed was significant in the first and third experiment, but there was no difference among the groups in the second experiment.

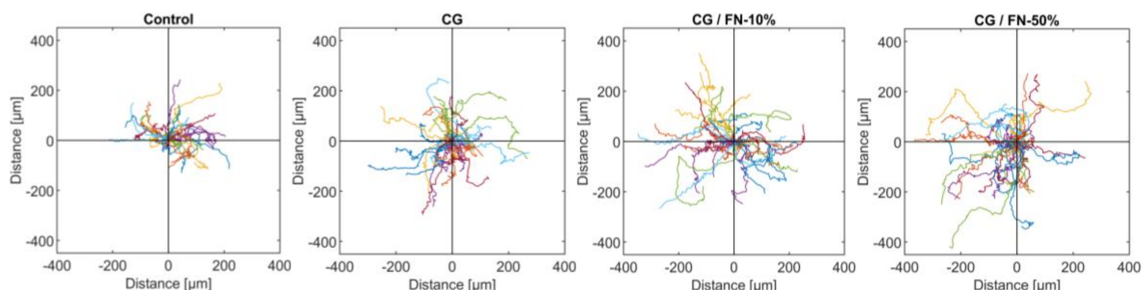


**Figure 3:** Cell traces (directional roses) performed in experiments.

In [7] it was also suggested that FN added to the collagen gel affects cell migration pathways *in vivo* and in culture. To verify this, an evaluation of the net distance travelled by the cell during the experiment, which reflects the directionality of the migration, can be performed. But the effect of FN on the direction of hMCS motility was not statistically confirmed in any experiment.

# 4 CONCLUSION

Collagen is the predominant extracellular matrix glycoprotein in most animals. Reconstituted collagen gels are commonly used in many standard in vitro 3D assays. This paper describes an experiment where collagen was used to simulate the pseudo-3D environment of an extracellular matrix. It was found that cells cultivated under a collagen gel layer migrate faster than in a medium, but cells did not migrate into the collagen. The addition of fibronectin to the gel also has a positive effect on the speed of cell migration. However, the addition of fibronectin did not affect cell migration pathways.

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