Report

Deformability cytometry an the impact of the doxycycline on the cells

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ABSTRACT

The mechanical properties of cells can be leveraged for the detection of pathologies. In recent years, there has been a notable advancement in techniques designed for the continuous mechanical characterization of large cell populations. One such technique is deformability cytometry, representing a microfluidic methodology that enables the simultaneous capture and assessment of the morphology and rheology of 1000 cells per second within a microfluidic channel. This technique induces cell deformation through hydrodynamic forces, eliminating the need for mechanical contact. Segmental images of the cells are recorded and can be subsequently utilized for in-depth analysis. Deformability cytometry can perceive modifications in the cytoskeleton, enabling the differentiation of cell cycle phases and the identification of subpopulations in the blood. The multiple parameters obtainable through deformability cytometry pose an analytical challenge that necessitates standardization.

INTRODUCTION

The emergence of flow cytometry dates back to 1970, and since then, it has become a widely used technique in biology. The principle of flow cytometry is that fluorescent markers are used to label cell structures, making them identifiable by laser excitation. The ability of these fluorescent markers can measure thousands of cells per second explains the technique's widespread use in recent decades, enabling the distinction of complex cell populations.

Fluorescent markers must be injected into the cells prior to the application of before applying these techniques. However, this process can be expensive, time-consuming and potentially damaging to the cells. This is why, in parallel with the development of this technology, the introduction of label free markers has been observed. Several types of scattered signals can be used as label free markers, for example to obtain cell characteristics such as size and refractive index. We will illustrate this later in this study, but there are advanced morphological and rheological parameters that contribute to more exhaustive cell characterisation. The rise of these techniques can be explained by the fact that certain mechanical properties of the cells can be used to identify pathologies. Another advantage of this technique is that label free markers can differentiate and split stem and progenitor cells from mature cells, which greatly facilitates studies. There are several measurement techniques using label free markers, including micropipette aspiration, optical stretcher and optical tweezers. However, these methods have a substantial disadvantage in that they can only process one cell per second, unlike microfluidic technology. The latter requires small volumes of fluid of the order of a micrometer to pass through channels capable of processing more than 50,000 cells per second. These methods also had a limitation in that they restricted time-dependent measurements and could potentially increase bias. For these reasons, laboratories have developed methods using microfluidics such as microchannel resonators, hydropipetting and deformability cytometry, which will be the subject of this study.

Deformability cytometry is a contact free stretching method based on the force generated by the extensional flow, inducing a non-laminar flow. This generates significant deceleration when the opposite flow meets it, generating high strains rates that deform the material in a few tens of microseconds. Finally, we are able to measure the aspect ratio and process approximately 2000 cells per second.

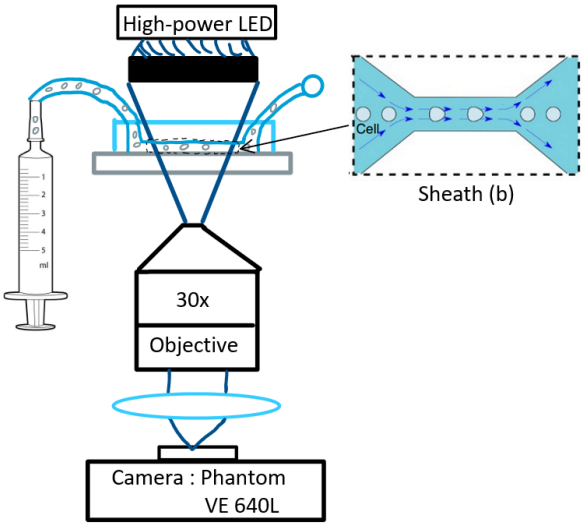
Cytometric deformability uses inertial focusing to align cells, enabling a wider range of cell sizes to be measured. However, this method involves considerable image refreshing, which generates huge amounts of data. This raises problems of data storage and processing. One solution to these problems is real time deformability cytometry, which uses a channel with a smaller diameter than the probed cells. The geometry and viscosity of the measurement buffer induce sufficient shear force to deform the cells so that they pass through the channels. With this method, up to 1000 cells per second pass through the channel and are recorded by high-frequency cameras, after which the data can be processed to extract cell parameters. The techniques used to do this will be explained later in this paper.

MATERIALS AND METHODS

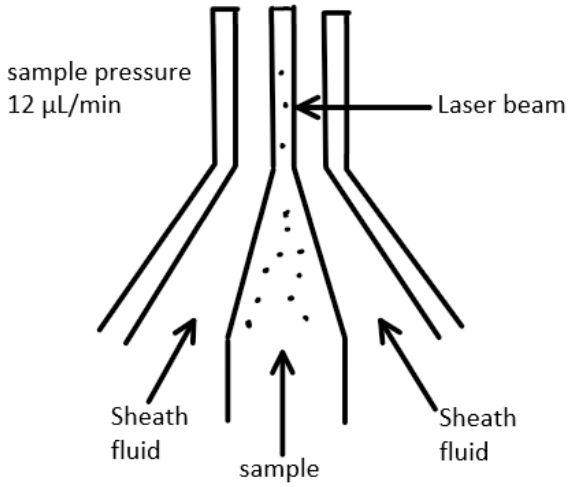
When describing the methodology, we drew heavily on the paper from Otto and colleagues (1), as we had no indication of how the experiment would unfold prior to obtaining the videos.

This is why we will assume that the experimental setup for acquiring the videos was relatively similar to that described in the previously mentioned paper. The experimental setup comprises a microfluidic chip made from poly(dimethylsiloxane). The microfluidic chip contains two reservoirs that are connected by a channel 300µm long and 30µm wide and deep, the value of the cross-section being relative to the size of the cell. According to Oliver Otto and colleagues(1), the cell size should be between 50% and 90% of the channel size to ensure that the shear gradients are large enough. Next, the microfluidic chip is connected to a syringe which will send the cells into the channel at a flow rate of 12µL/min. The shape of the sheath allows the flow of cells to be centred laterally and vertically in the channel. The analytical model used to find the flow profile in this microfluidic channel consists of using the hydrodynamic stress on the surface of the object and then quantifying the deformation according to the theory of linear elasticity.

The forces acting on the cells are firstly a force resulting from the normal pressure distribution on the spheres, which acts essentially in the direction of flow. On the other hand, we also have a viscous shear force acting against our first force and therefore in the opposite direction to the flow. In part B of the study, we will study the deformability cytometry technique in order to analyse the effects of the expression of a particular gene, which could potentially have an impact on cell mechanics. We will therefore be working with a particular type of cell that endogenously expresses ME480, the gene of interest. To carry out our first test, we will use cells possessing a gene that once activated will produce a protein that masks the expression of ME480. These cells will be labelled CellA_ShME480. It is important to note that the expression of the regulatory gene will be activated following exposure to doxycycline, a molecule from the cycline family used mainly as an antibiotic drug. These CellA_ShME480 cells were exposed to this molecule for 6 days prior to the measurements. In part C of the study, we will also consider a second cell type, B cells that do not endogenously express the ME480 gene. So we end up with four different categories of cell : 1. cellA_ShME480: the same cell line that was used in part B. 2. CellA_GFP : cellA cells are transfected with a gene that produces a green fluorescent protein when the cells are exposed to doxycycline. 3. CellB_ME480 : cellB cells that are transfected with ME480 gene which gets expressed when the cells are exposed to doxycycline. 4. CellB_GFP : cellB cells are transfected with a gene that produces a green fluorescent protein when the cells are exposed to doxycycline.



(a) Experimental setup



(b) Sheath schematics

Figure 1: Schematics of the experimental setup(a)(1) and a more precise schematics of the sheath(b)

Microfluidic experiments

In the context of this experimental setup for deformability cytometry, meticulous attention was given to defining flow cytometry and image acquisition parameters, ensuring robust and reproducible datasets. For the flow cytometry component, a cell sample flow rate of 4 µL/min, coupled with a sheath flow rate of 12 µL/min, was employed. The microfluidic channel, with dimensions of 300x30x30 µm (length x width x depth), provided a controlled environment for the passage of cells, subjecting them to predictable stresses for subsequent deformation analysis. Image acquisition was conducted using a Phantom VE 640 L camera, capturing phase-contrast images with an exposure time of 1µs at an impressive frame rate of 10,000 fps. Each recorded movie comprised 356,953 frames, with a pixel size of 10x10 µm and a magnification of 30x. The resulting images, with dimensions of 256x128 pixels, serve as the foundation for our comprehensive analysis. These meticulously selected parameters not only facilitate the quantification of mechanical properties but also ensure

the reliability and depth of the acquired datasets, laying the groundwork for a rigorous investigation into the mechanics of the cell population under study.

Image processing

To analyze the images obtained in the previous step, it is important to choose the images of interest. Since there is not a cell on each frame of the video, the first objective is to delete the empty frames. This consequently reduces the size of the file and the computational time. To perform this reduction, a basic algorithm is utilized. First, the frame is cropped to remove the sides of the tube visible in the video. Then, a threshold on the image is performed to obtain a mask. If the white part of the mask is sufficiently large, the frame is kept. Otherwise, the frame is removed. Finally, a file is obtained with only deformed cells in it.

Once the file is cleaned, a segmentation is performed using the `Omnipose` library (2) in Python. The pretrained model used was the `cyto2` model. This library was chosen to obtain the best segmentation as possible, even tough it meant an lot higher computational cost. The output of this image processing is a binary mask separating the cell from the background. The mask is then analyzed to get different features of each cell. The feature are the following :

$$Circ = \frac{4\pi \cdot A}{P^2} \qquad Round = \frac{1}{AR} = \frac{h}{w} \tag{1}$$

Here $Circ$ is the circularity of the cell in terms of the area A and the perimeter P of the cell. $Round$ stands for roundness of the cell which was defined to be the invert of the Aspect Ratio which is in terms of the height and the width of the bounding rectangle of the cell in this case (3). To obtain these values, the computer vision library `OpenCV` was used, especially the functions `cv2.findContours()`, `cv2.contourArea()` and `cv2.arcLength()`.

Data analysis and statistical methods

The data extracted from the image processing are collected and stored in an excel sheet. With the help of the `panda` library, it is then possible to extract the data of interest and store them in `numpy` arrays. Once the data is stored in an array, it is ready to be processed. The mean and standard deviation are computed. The outliers values are managed by removing values that are more than one standard deviation away from the mean value. Furthermore, if more than one cell is detected during the imaging process, the values extracted from that frame are removed to avoid treating corrupted data. Boxplots present a more comprehensive way to plot the resulting cleaned data. They allow to visualise the distribution of the data more rapidly and easily, while giving perspective to the mean value. The previously computed mean value also figures on the boxplot to give additional information of the data.

RESULTS

Part A

The input file `CellA_noDoxycycline.avi` was heavy (11.0 Go). Thus, it was necessary to process it. The aim was to delete the redundant frames by only keeping the frames where a cell was in, as explained in *Image processing* section. The obtained output `CellA_noDoxycycline_processed.avi` is much lighter (16.9 Mo). There are 17′435 over 356′953 frames kept after processing.

Part B

It can be seen that for the 35 first minutes since detachment, the circularity as well as the roundness of the cells change and increase to reach a new steady equilibrium. From 35 minutes to 48 minutes, the circularity and the roundness stay the same and stabilise around 0.938 for the circularity and 0.72 for the roundness

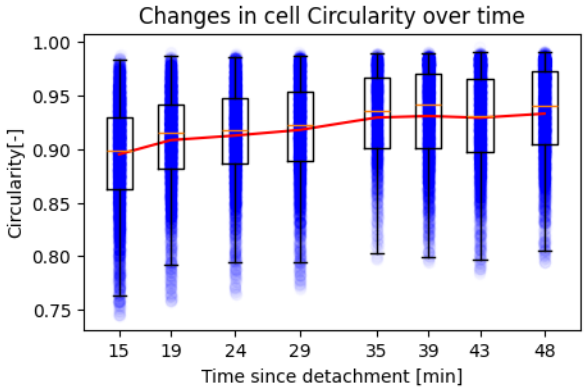
When looking at the data recorded at different time points, it can be observed that the circulation and roundness of the cells increase in time. This indicates that during the early stages of the experiments, the cell tends to be more easily deformed. Usually in an elongated shape. As time goes, the increasing of roundness and circulation underline that the cell's shape resemble increasingly to a sphere. The cell is therefore less affected by the microfluidic jet which we can relate to its rigidity increasing with time.

As of now, stress–induced deformation for stiffer cells has not been demonstrated with the deformability cytometry (DC) technique. Since we have seen in our study that the stiffness seems to increase in time, advances in the field of DC need to be made in order to characterize these stiffer cells more accurately. Pharmacological tests would need to provide higher stresses and thus higher velocities methods to improve the overall accuracy of the said tests.

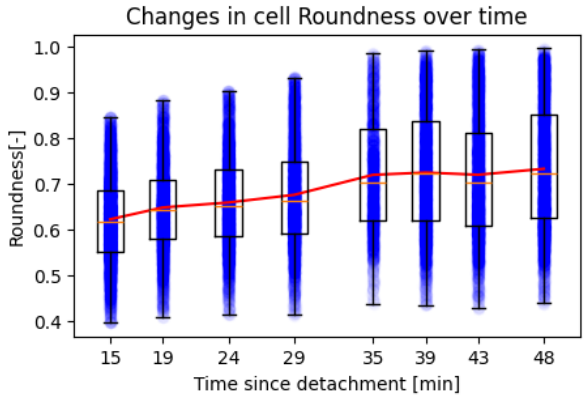
Part C

There are two types of cells studied here : cells A or cells B. For each type, a gene (ME480) is expressed or not. It can be observed in Fig. 3a that the circularity average is quite the same for each type of cells. But the standard deviation of data is larger when the cells are transfected by the ME480 gene. Concerning the roundness in Fig. 3b, cells A and B have distinct results. Cells A have a larger roundness than cells B. The effect of ME480 gene on the roundness is clear. It reduces the roundness of the cells independently of their type. So, it means that the ME480 gene affected globally the roundness of each cells but affected only partially their circularity.

In terms of algorithm, it was chosen to remove the frames with more than one cell. This way, the resulting data are readable. Another reduction of data was performed to remove the outliers. The disadvantage of this reduction of frame is that a non negligible part of data are lost. After segmentation, the percentage of frames kept for each cells type are summarized

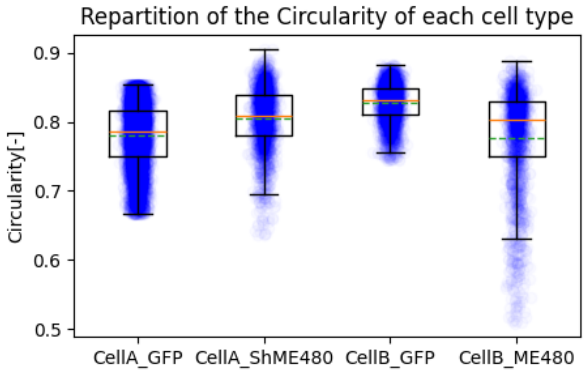


(a) Circularity

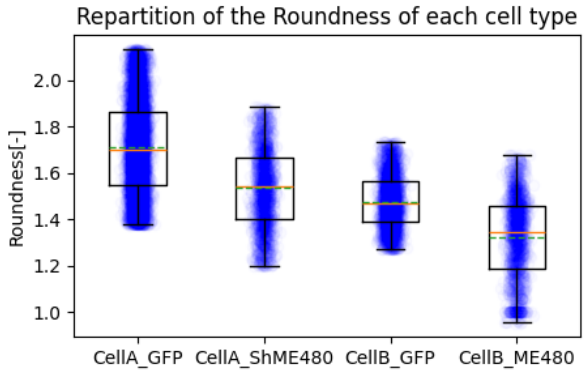


(b) Roundness

Figure 2: Results for Part B tasks consisting of analyzing (a) the circularity and (b) the roundness of the CellA_ShME480 along the time.



(a) Circularity



(b) Roundness

Figure 3: Results for Part C tasks consisting of analyzing (a) the circularity and (b) the roundness of different types of cells : CellA_GFP, CellA_ShME480, CellB_GFP and CellB_ME480.

in the following table :

Table 1: Percentage of frames kept after data cleaning

Cells type	Frames kept [%]
CellA_GFP	76.24
CellA_ShME480	54.56
CellB_GFP	65.31
CellB_ME480	39.35

Since the segmentation was performed using machine learning, the runtime is consequent. To improve it, a possibility is to change the algorithm of segmentation. It was tried to use a simple threshold but it was impossible to obtain a good analysis of the contours of the cells and thus, the circularity and roundness were biased.

DISCUSSION AND CONCLUSION

Despite clear results on the effect of the expression of the ME480 gene, multiples sources of error can be discussed. First, computer vision is not a perfect science and errors are introduce during the segmentation. If the segmentation is not as perfect as possible, all the data used after are not really correct. It is important to have the right algorithm of segmentation. Then, the suppression of frames with more than one cell and of outliers leads to a loss of data. This loss can be non negligible and maybe it would say something in terms of results. As shown in Table 1, the larger loss of frames is for the cells where the ME480 gene is expressed. This is correlated with the fact that the standard deviation is larger for cells with the ME480 gene. Since the standard deviation is larger, then there will be more outliers. The last source of error in this study is that it was chosen to only extract two features. These features are sufficient to distinguish cells A and B but to clearly demonstrate the effect of the ME480 gene, it can be interesting to analyze more features to see if the ME480 gene has other effects on the mechanics of the cells A and B and if these effects are different with respect of the cell

type.

In conclusion, the effect of the ME480 gene was tested. The results showed that this specific gene tends to reduce the roundness of the cells whatever their type (A or B) without changing their average circularity. It is possible to improve the analysis of data by considering other features or to improve the runtime of computation by performing another segmentation algorithm.

REFERENCES

1. 2015. Real-Time deformability cytometry : on-the-fly cell mechanical phenotyping https://www.nature.com/articles/nmeth.3281.

2. Cutler, K. J., 2022. Omnipose: a high-precision morphology-independent solution for bacterial cell segmentation https://doi.org/10.1038/s41592-022-01639-4.

3. ImageJ documentation https://imagej.net/ij/docs/menus/analyze.html#set.

SUPPLEMENTARY MATERIAL

- Jupyter notebooks computing Parts A, B and C, respectively named Ajouter les noms des .ipynb
- Video file output from Part A `CellA_noDoxycycline_processed.avi`
- Excel files output of the segmentation in Part C : `CellA_GFP_data.xlsx`, `CellA_ShME480_data.xlsx`, `CellB_GFP_data.xlsx` and `CellB_ME480_data.xlsx`