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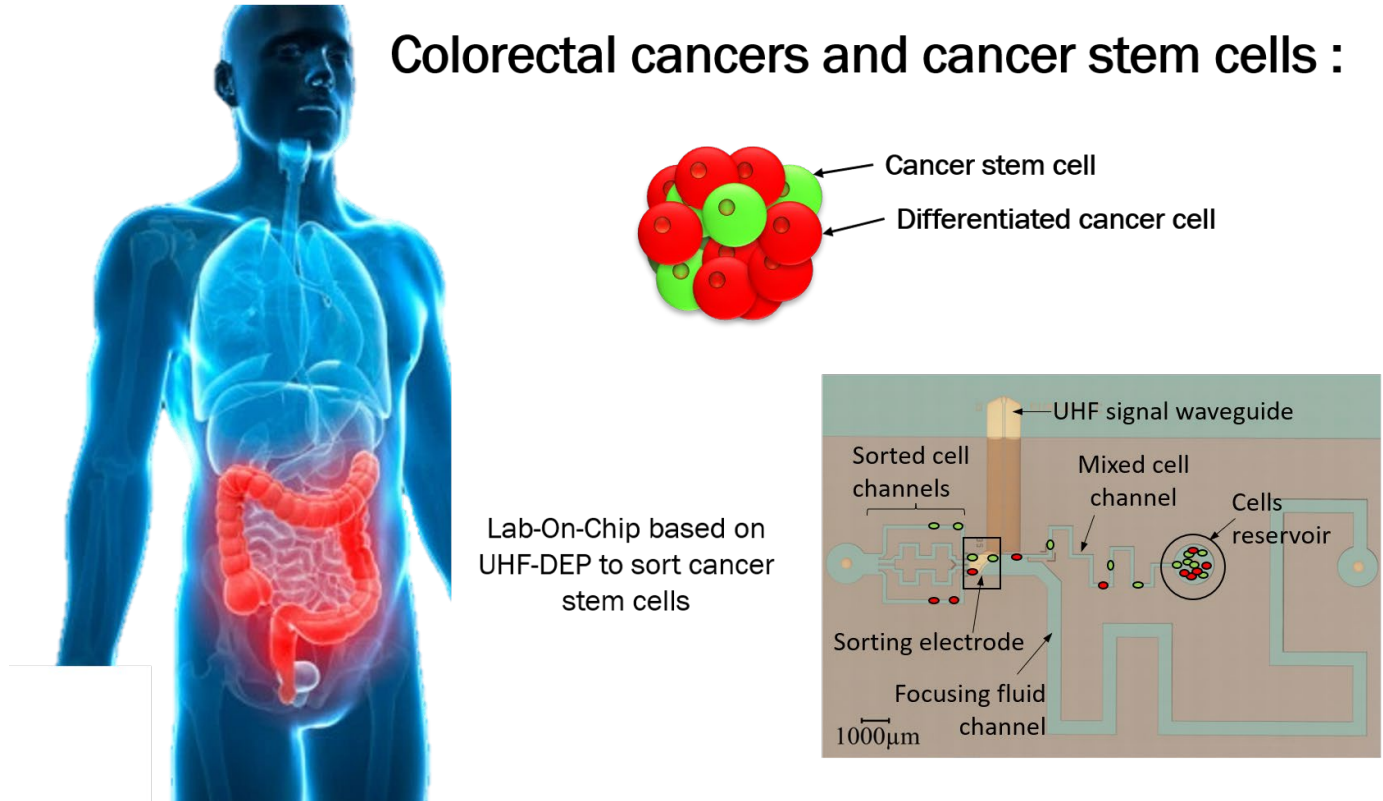
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Lab-On-Chip UHF-Dielectrophoretic Cytometer for Colorectal Cancer Stem Cells Sorting

E. Lambert¹, E. Barthout², T. Provent¹, R. Manczak¹, S. Saada², B. Bessette², M. Mathonnet³, F. Lalloué², C. Dalmay¹, A. Pothier¹

Colorectal cancers and cancer stem cells :



Take-Home Messages

- **Innovative features:** The proposed approach presented in this paper, is based on label-free Ultra-High Frequency dielectrophoresis (UHF-DEP). This method allows to investigate intracellular content by exploiting its interaction with an electric field to establish the dielectrophoretic signatures of cells, based on their dielectric properties. An UHF-DEP cytometer is hence developed based on this innovative method in order to sort cancer stem cells (CSCs) among a heterogeneous cell population.
- **Main conclusion:** This paper demonstrates that UHF-DEP can be exploited to sort and isolate the targeted cell population with the developed lab-on-chip dielectrophoretic cytometer.
- **Targeted application:** In many cancers and especially in colorectal cancer (CRC), CSCs are known to be the most aggressive and resistant cells in the tumor. Due to the lack of specific markers for detecting the occurrence of such cells as a prognosis of a potential therapeutic failure, new approaches and technologies allowing the characterization of such cells are required. Apostream, a commercial system, operating at low DEP frequency regime has been especially developed for liquid biopsies to isolate circulating tumor cells (CTCs) from patient peripheral blood. However, in the frame of solid cancer investigation, it has been previously showed that UHF-DEP is discriminant toward CSCs and can be more relevant to use in order to isolate such cells from a solid tumor.
- **Breakthrough:** UHF-DEP is sensitive to intracellular content and can selectively electro-manipulate biological cells related to their intrinsic dielectric specificities and may be relevant and useful to the biological diagnosis and pathology.

Lab-On-Chip UHF-Dielectrophoretic Cytometer for Colorectal Cancer Stem Cells Sorting

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Abstract - In this paper, we introduce a method to exploit ultra-high dielectrophoresis (UHF-DEP) with a microfluidic radio frequency device able to sort biological cells by deflecting the trajectory of the targeted population in a continuous flow. This study highlights the capability of a high frequency lab-on-chip implemented UHF-DEP cytometer to isolate cancer stem cells (CSCs). Actually, CSCs sorting by an efficient, fast and low-cost method remains an issue. Above 20 MHz, the intracellular dielectric properties of cells determine the dielectrophoretic behavior of cells and is then very sensitive to the cell cytoplasm content. The proposed cell sorting principle exploits the combination of the fluidic drag and the repulsive dielectrophoretic forces in order to isolate CSCs hidden within differentiated cancer cells. We previously demonstrated the UHF-DEP behavior of cells, as a new type of electromagnetic biomarkers to discriminate cancer stem cells among a tumor population from colorectal cancer cell line.

Keywords — Biological cell manipulation, Colorectal cancer stem cells, Label-free cell sorting, Microfluidic biosensors, UHF dielectrophoresis

I. INTRODUCTION¹

Despite the improvement of diagnosis and therapeutics, relapse and tumor recurrence still occur in many cancers. It remains all along nowadays a major issue especially in the therapeutic management of colorectal cancer (CRC). The presence of cancer stem cells (CSCs) able to resist to radio- and chemo-therapeutic treatments could explain such treatment failures. The undifferentiated phenotype of CSCs [1] and their presence inside the tumor mass, despite their very low number, have been correlated with a high risk of minimal residual disease, able to regenerate the tumor mass, which would favor disease recurrences [2]. CSCs reflect the aggressiveness of the tumor in terms of proliferation, invasion and colonization. Their presence within the tumor or in the periphery represents a poor prognostic factor for the patient. Therefore, CSCs' detection from tumor biopsy is a challenge to establish a reliable prognostic and improve the therapeutic care of solid cancers. Currently, in order to isolate CSCs from the tumor, biologists use fluorescence activated cell sorting (FACS) which is based on the labelling of target cells, using antibodies coupled to a fluorochrome. Although FACS is a

powerful sorting and analysis technic, currently commonly used immuno-markers in oncology such as CD133, Nanog, Sox2 and Oct4 stay generic labels and it has been demonstrated that they can also be expressed by differentiated cancer cells [3]. Actually, the lack of CSCs specific biomarkers remains an issue, especially since they represent less than few percent of cells in the tumor mass [4], making their isolation even more complex. As a result, to support the evidence of CSCs presence, biologists must analyze a wide panel of biomarkers, with expensive and time-consuming staining steps. Moreover, this approach should be complemented by functional tests such as clonogenicity [5], which require up to 20 to 40 days of process to establish the ability of suspected CSCs to renew a tumor. That is why, researchers have started to develop alternative label-free cell sorting methods exploiting effects on single-cells of motion forces within a microfluidic environment, which can be implemented for clinical purposes.

In this paper, we propose an alternative and complementary cell discrimination approach based on dielectrophoresis (DEP) which exploits the intrinsic dielectric properties of biological cells [6]. This technique presents an important interest as it is a low-invasive sorting method which prevents cells from damages by limiting mechanical stress. Apostream, a commercial system, already exploits low DEP frequency regime to isolate circulating cancer cells (CTCs) from for patient liquid biopsies (blood) [7]–[9]. However, in the frame of solid cancer investigations, it has been established that UHF-DEP might be more discriminant toward CSCs and can be relevant to use in order to isolate such cells from a solid tumor [10], [11]. The originality of this paper especially relies on the used frequency range to sort cells: a high frequency regime

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(between 300MHz and 3GHz). Indeed, the proposed UHF-DEP cytometer allows to investigate intracellular content by exploiting its interaction with an electric field to establish the dielectrophoretic signatures of cells based on their dielectric properties. Significant differences of the dielectrophoretic signatures observed between cell types are correlated to phenotypical difference and could be exploited to sort and isolate treatment resistant CSCs within a tumor.

To do so and to demonstrate the feasibility of the proposed approach, we will exploit the specificities of the dielectrophoretic signatures previously obtained in [10], reminded in Fig.1a, where a colorectal cancer cell line SW620 has been cultured in two different conditions: one with a large majority of differentiated cancer cells (Normal Medium culture condition) and another enriching the cell population in CSCs (Define Medium culture condition). Statistics are reported in Table I. According to Fig.1b, the SW620 NM cell size mean is $12.4 \mu\text{m} \pm 2.1 \mu\text{m}$ and SW620 DM is $11.9 \mu\text{m} \pm 1.9 \mu\text{m}$.

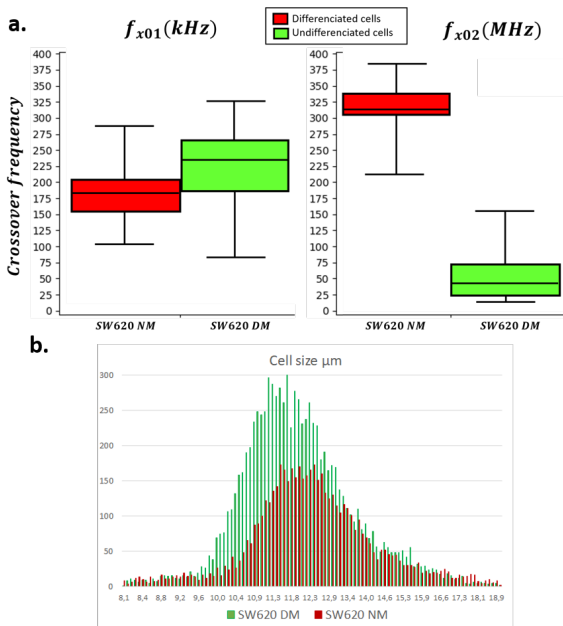


Fig. 1. (a) Box plot representation of the two crossover frequencies f_{x01} and f_{x02} for SW620 NM and SW620 DM cell cultures. (b) SW620 NM and DM cell size repartition

TABLE I

STATISTICAL RESULTS FOR THE TWO CROSSOVER FREQUENCIES F_{x01} AND F_{x02} MEASURED ON CELL LINE SW620

| Culture condition | Crossover frequency | Mediane value | Standard deviation |
|-------------------|---------------------|---------------|--------------------|
| NM | f_{x01} | 184 kHz | 41 kHz |
| DM | | 234 kHz | 68 kHz |
| NM | f_{x02} | 313 MHz | 50 MHz |
| DM | | 44 MHz | 40 MHz |

II. HOW DIELECTROPHORETIC CYTOMETERS OPERATE

A. Working principle of the DEP cytometer

Dielectrophoresis is a physical phenomenon describing the motion of a polarizable particle, such as biological cells

in a non-uniform field. The induced force is given in the following equation:

$$F_{DEP} = 2\pi\epsilon_m r^3 \text{Re}[f_{cm}(\omega)] \nabla E_{RMS}^2 \quad (1)$$

where r is the particle radius, ϵ_p^* and ϵ_m^* respectively the complex permittivity of the cell and the medium, ω is the angular frequency of the applied electric field, E_{RMS} is the root mean square value of this electric field and $\text{Re}[f_{cm}(\omega)]$ the real part of the Clausius-Mossotti (CM) factor, which is described as follow [6]:

$$f_{cm}(\omega) = \frac{\epsilon_p^* - \epsilon_m^*}{\epsilon_p^* + 2\epsilon_m^*} \quad (2)$$

The CM factor describes the polarization state of the cell in the suspension medium and modulates the orientation and the magnitude of the DEP force. As shown in Fig. 2, when the real part of the CM factor is negative, the biological cell presents a nDEP behavior and is repulsed toward weak electrical field area (cyan & blue areas in Fig.3c). Otherwise, when the real part of the CM factor is positive, a pDEP behavior is observed and the cell is attracted to high electrical field magnitude (red area in Fig.3c). Two crossover frequencies, f_{x01} at low frequency and f_{x02} at high frequency, appear from pDEP to nDEP transition and occur when the DEP force cancels out. They are referred as the “dielectrophoretic signatures” of the cell.

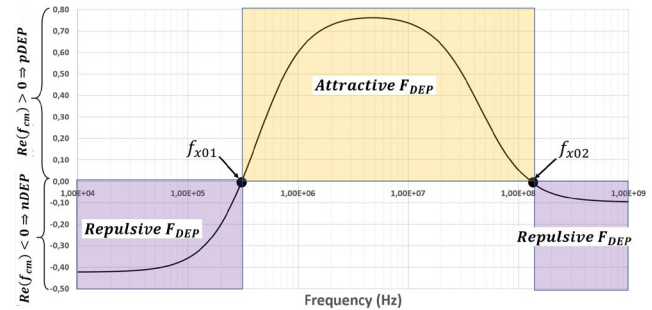


Fig. 2. Plot of the real part of the CM factor of a biological cell with its two dielectrophoretic signatures f_{x01} and f_{x02} .

Both crossover frequencies depend on biological cell’s specificities [11]. At low frequency (from 10 kHz to 1 MHz), the plasma membrane of the cell acts as an insulator and the electromagnetic wave is more sensitive to the dielectric properties (conductivity and permittivity) of the membrane. Moreover, f_{x01} depends also on the cell shape, size and morphology. Low frequency regime has been widely used to separate cells of different sizes [7]–[9], [12] and to separate viable from non-viable cells [13], [14]. On the contrary, at ultra-high frequencies, the plasma membrane is short-circuited and becomes transparent to the electromagnetic wave which can then probe and interact with the intracellular content. Consequently, the crossover frequency f_{x02} depends mainly on the dielectric properties of the cell’s content. In this way, we have previously demonstrated that the UHF-DEP signature appears as a relevant biomarker for CSCs discrimination and detection among a heterogeneous cancer cell population [10], [11], [15], [16]. Hence, biological cells can be individually electro-manipulated by the motion induced by the DEP force. The resulting motion can be modulated mainly by the DEP force magnitude which can

change from a cell to another. On this basis, we will propose to experiment a sorting method to deviate and to isolate the sub-population of CSCs from a heterogeneous cell suspension by using a microfluidic implemented cytometer based on UHF-DEP.

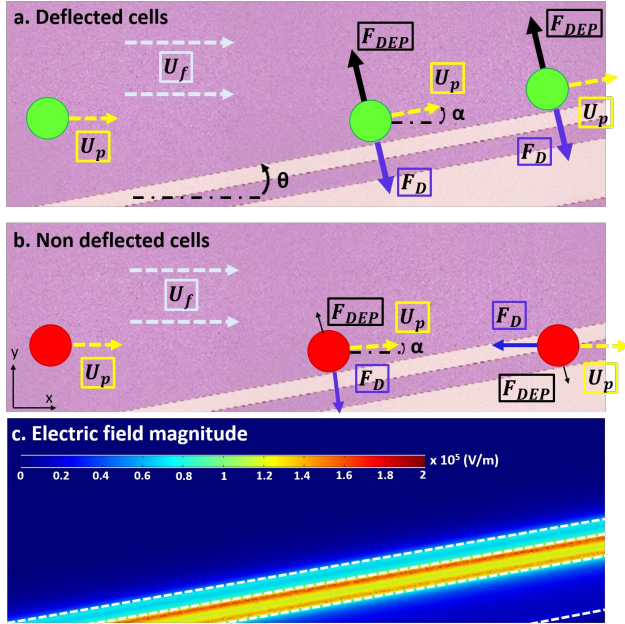


Fig. 3. (a) and (b) Working principle of selective cell trajectory deviation related to intrinsic cell dielectric intracellular properties. With F_{DEP} is the DEP force, F_D the Drag force, α the deflection angle of the cell trajectory and θ the electrodes slope. (c) COMSOL Multiphysics numerical simulation of the electric field b considering a applied voltage of 5Vpp between electrodes.

The working principle of the UHF-DEP cytometer is based on a balance between two forces: the repulsive UHF-DEP and flow-induced forces. Indeed, in the microfluidic channel, the cell flow is continuous and thus the cell will follow the trajectory of the fluid stream. When the cell arrives near the electrodes, it enters under the influence of two forces in competition: the repulsive DEP force and the drag force induced by the change of cell speed U_p related to the speed U_f of the fluid flowing in the microchannel. Indeed, the repulsive effect of the DEP electric field induces a modification of the cell's velocity whereas the speed of surrounding suspension medium in which it is immersed, stays the same. The drag force will exist until this difference of speed disappears. It is expressed as follow [17]:

$$\vec{F}_D = 6\pi\mu r^2(\vec{U}_f - \vec{U}_p) \quad (3)$$

where μ is the dynamic viscosity of the fluid and U_p the cell speed. And U_p can be expressed in our case as following : $U_p = U_f + \Delta U$ where ΔU is the slight cell slow-down generated by the DEP force.

Notice that operating with repulsive DEP, i.e. in nDEP regime, allows the electric field to deflect the cells of interest from electrodes while limiting the phenomena of cell adhesion on the electrodes, most of the time amplified by a pDEP regime. As the selected sorting frequency f_i influences the intensity of the response of the biological cell to be more or less repulsed, different operating conditions can be set up. When the fluidic and electric forces at f_i are balanced, the repulsive DEP force is strong enough to repel the cell toward

weak electric field area (Fig. 3a). When the fluidic force is stronger than the electric one, the DEP force is no longer enough strong to repel the cell and the fluid drives the cell along its initial trajectory (Fig. 3b).

B. Taking advantage of the cells' UHF-DEP signatures to sort them

As described above, the electromagnetic cytometer, once biased with a UHF continuous wave (CW) signal set at the selected sorting frequency f_i , will operate mostly in the repulsive regime. The biological cells are either strongly repelled by the electrodes, or too weakly repelled and then, cross the electrode area pushed by the flow. In this way, a first lab-on-chip cytometer prototype has been designed (Fig. 5). It used on an RF coplanar line as deviating electrode that carries out the DEP signal into the main channel while forming different angles with it. Nevertheless, during the following sections, we will focus on a case of a single angle electrode section. When the cell is submitted to a strong repulsive force and so is deflected by the electrodes (Fig.3a), it follows a uniform rectilinear motion, parallel to the electrodes. The resulting force applied on the cell is zero because of a balance between the electrical and fluidic forces, giving the following equality:

$$\vec{F}_{DEP} = \vec{F}_D \quad (4)$$

The change in the cell's velocity vector initiated by the DEP force causes its trajectory to be deflected with an angle α with respect to the direction of the fluid. The projections of (4) on the x axis and y axis give the following equations:

$$\begin{cases} -2\pi\epsilon_m * Re[f_{cm}] * r^3 * \nabla|E_{RMS}|^2 * \sin(\theta) = 6\pi\mu r * (U_f - U_p \cos \alpha) \\ 2\pi\epsilon_m * Re[f_{cm}] * r^3 * \nabla|E_{RMS}|^2 * \cos(\theta) = -6\pi\mu r * U_p \sin \alpha \end{cases} \quad (5)$$

where α is the deflected angle of the cell's trajectory and θ the electrodes slope. The resulting equation system (6) allows to determine the velocity of a cell presenting a given radius r and a CM factor f_{cm} :

$$\begin{cases} U_p \cos \alpha = U_f + \frac{\epsilon_m * Re[f_{cm}] * r^2 * \nabla|E_{RMS}|^2 * \sin(\theta)}{3\mu} \\ U_p \sin \alpha = -\frac{\epsilon_m * Re[f_{cm}] * r^2 * \nabla|E_{RMS}|^2 * \cos(\theta)}{3\mu} \end{cases} \quad (6)$$

For our purpose, the cell will be efficiently deflected for a minimum deflection angle $\alpha_{min} = \theta$, that will ensure to continuously repel the cell along the electrodes while the fluid drives it to flow within the microchannel. Hence, by dividing the two equations by each other, we can calculate a "critical" CM factor f_{cm} which represents the minimal $Re[f_{cm}]$ value to be owned by a biological cell able to follow the slope of the electrodes and be sufficiently deflected:

$$f_{cm}^c = -\frac{3\mu U_f \sin(\theta)}{r^2 \epsilon_m \nabla|E|^2} \quad (7)$$

It means that when the absolute value of a biological cell $Re[f_{cm}]$ is greater than the absolute value of the critical CM factor, the cell trajectory will be clearly deflected (Fig. 3a), otherwise the cell will cross the electrodes with a negligible impact on its trajectory (Fig. 3b). Actually, the critical CM factor does not depend on the frequency of the applied signal but only on the geometry of the electrodes, the fluid flow velocity and the electric field gradient magnitude. These parameters can be tuned to modulate the critical CM factor

in order to select which cell sub-population can be isolated and separated from the whole population.

During previous studies, we established that the dielectrophoretic signature f_{c02} of CSCs are generally lower than those of differentiated cancer cells. Thus, we expect that CSCs could be strongly repulsed by the electric field generated by electrodes if they are biased with a UHF-DEP signal set at a frequency higher than their second crossover frequency of the targeted cells. Our objective is to deflect the trajectory of a large majority of CSCs while differentiated cancer cells are not repulsed enough by the electric field and should cross above the electrodes (Fig. 3). Hence, we must satisfy the following conditions with f_i the sorting frequency:

- $\text{Re}[f_{cm}]_{csc} < f_{cm}^c < 0 \quad @f_i$
- $f_{cm}^c < \text{Re}[f_{cm}]_{differentiated} < 0 \quad @f_i$

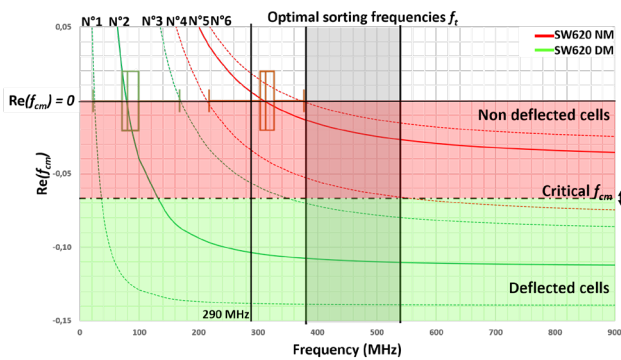


Fig. 4. Theoretical plot of the real part of the CM factor as a function of the frequency and according to the measured dielectrophoretic signatures for the SW620 NM (red curves) and SW620 DM (green curves) from Fig.1. The critical f_{cm} is determined over a range of possible values for an angle θ of 10° , considering the electrodes are biased with a signal magnitude of 37.7 dBm and a fluid flow velocity in the microchannel $7 \text{ mm}\cdot\text{s}^{-1}$.

In Fig.4, we approximated the evolution of $\text{Re}[f_{cm}]$ for the two SW620 populations by relating the dielectrophoretic signatures from the box plot in Fig. 1 [10] and possible values of CM factor. It is necessary to specify that because the difficulty to access to accurate measurements of the dielectric parameters on individual biological cells, we have made several assumptions. We used a one-shell model for the cell with the plasma membrane and a homogeneous cytoplasm, neglecting nucleus and organelles. For the present example, the cell is assumed to present a spherical shape of radius $6\mu\text{m}$ and smooth morphology. Moreover, we have imposed values for the conductivity of the internal medium, computed the corresponding relative permittivity according to the range of measured transition frequencies for two kind of colorectal cancer cells: differentiated (NM) and undifferentiated (DM) from SW620 line (as detailed in ‘‘Cell culture and preparation’’). The values considered are reported in Table.II. One should also notice that to obtain such plot, the same radius of $6\mu\text{m}$ has been considered for each cell as well as the considered membrane conductivity and permittivity, values extracted from [18] which $5.02\cdot 10^{-5} \text{ S/m}$ and $6.12\epsilon_0 \text{ F/m}$ for a 5 nm thick membrane considered. In practice, the impact of the cell natural heterogeneity will result in more or less translating the

curves regarding frequency.

TABLE II
INTRACELLULAR DIELECTRIC PROPERTIES CONSIDERED TO PLOT THE REAL PART OF THE CLAUSIUS-MOSSOTTI FACTOR IN FIG.4

| Curve number of Fig.4 | Intracellular conductivity | Intracellular permittivity |
|-----------------------|----------------------------|--------------------------------|
| N°1 | 0.1 S/m | $49.7 \epsilon_0 \text{ F/m}$ |
| N°2 | 0.3 S/m | $54.68 \epsilon_0 \text{ F/m}$ |
| N°3 | 0.6 S/m | $59.3 \epsilon_0 \text{ F/m}$ |
| N°4 | 0.72 S/m | $61.34 \epsilon_0 \text{ F/m}$ |
| N°5 | 0.77 S/m | $69.62 \epsilon_0 \text{ F/m}$ |
| N°6 | 0.8 S/m | $72.01 \epsilon_0 \text{ F/m}$ |

One can notice that from 290 MHz, it should be possible to deflect a maximum of cells presenting a stem cell phenotype (SW620 DM). However, in this frequency range, a majority of cells presenting a differentiated phenotype (SW620 NM) are in pDEP regime and could adhere to the electrodes, disturbing the sorting with risk of cell cluster formation. As a result, the optimal sorting frequency range to have the greatest selectivity is estimated between 390 MHz and 430 MHz. Indeed, it is expected that a large part of SW620 DM population is deflected while the trajectory of differentiated cells remains the same.

Two prototypes have been developed based on the theory of the critical CM factor, one with four outlets in order to demonstrate the ability of the possible deflection and another one with two outlets to collect cell after sorting.

III. INFLUENCE OF THE CRITICAL CLAUSIUS-MOSSOTTI FACTOR ON CELL’S DEFLECTION

A. DEP cytometer design n°1

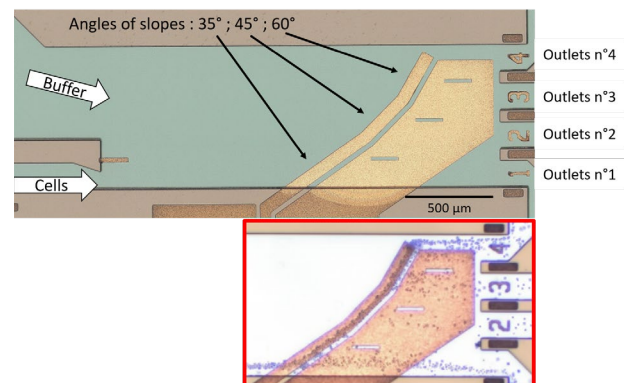


Fig. 5. Sorting area of the prototype n°1 of the dielectrophoretic cytometer. The implemented electrodes include three different sections to separate and guide cells into four outlets. The red frame illustrates the typical cell trajectory change occurring during a sorting experiment.

The first UHF-DEP cytometer design proposed (Fig. 5) has been designed in order to study the effect of the critical CM factor on the biological cell’s deflection. To do so, a dedicated electrode has been designed with three different sections with increasing slopes (35° , 45° and 60°). Hence, such UHF-DEP cytometer would be able to separate a cell suspension into four subpopulations driven at the end of the device where four outlets have been implemented to collect sorted cells [19].

B. Results

To evaluate the ability of the prototype to operate, experiments were performed on U87-MG cell culture known to host cells with high biological heterogeneity translating in quite dispersive dielectric properties [11]. As a first step, the aim is to study cells deflection according to the variation of the critical CM factor. To do so, cell's trajectories have been recorded using an automated image capture system (CV-X470F sensor from Keyence). This device can track and count particles at real time during experiments. It has been used and configured to count the number of cells going to each of the four outlets during an experiment of 30 min run in continuous flow.

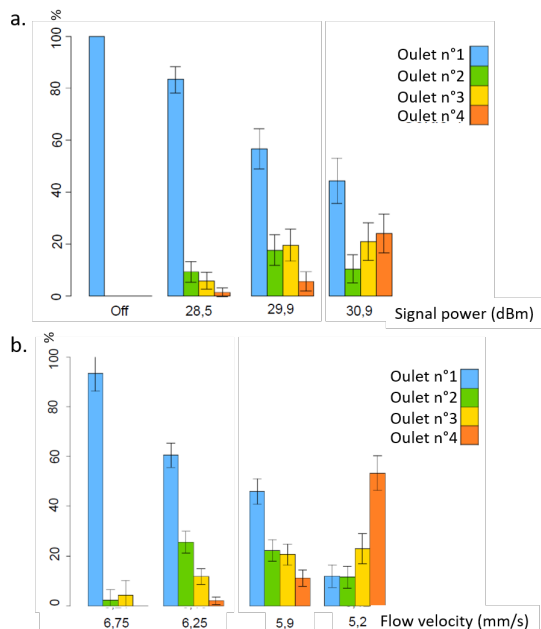


Fig. 6. Collected cells distribution at each sorting system outlets as function of (a) the signal power at constant speed flow of 4.7 mm.s^{-1} ; (b) the flow velocity at constant signal power of 29.9 dBm . The signal frequency was 500 MHz . The bars represent the standard error of mean.

As a first step, the cell suspension has been injected at a constant flow velocity of 4.7 mm.s^{-1} ($11 \mu\text{L}/\text{min}$ flow rate for this device). The electrodes were biased with a variable electric field magnitude which can be settled by a tunable delivered power of the radiofrequency generator: 28.5 dBm , 29.9 dBm and 30.9 dBm set at a frequency of 500 MHz (Fig. 6a). By the increase of the electric field magnitude, the DEP force magnitude increases too and the absolute value of the critical CM factor decreases. Thus, we do expect that a larger number of cells should be deflected. As illustrated in Fig. 6a, cells are deviated only when the electric field is switched on, otherwise following their initial trajectory they are all collected at the first outlet of the cytometer. Experimentally as expected, with the increase of the electric signal power, one can notice that more and more cells are deflected. Indeed, at 28.5 dBm , only 17% of the observed events react to the electric field and are deviated to the outlets n°2, n°3 and n°4. Whereas at 30.9 dBm , more than 50% of observed cells are deflected.

Secondly, the cell suspension has been injected at a variable flow velocity: 5.2 mm.s^{-1} , 5.9 mm.s^{-1} , 6.25 mm.s^{-1} and 6.75 mm.s^{-1} . The electrodes were biased with a constant magnitude DEP signal of 29.9 dBm set at 500 MHz (Fig. 6b). By increasing of the flow velocity, the absolute value of the critical CM factor increases too. Thus, we do expect that less cells can be deflected by the electric field. As shown in Fig. 7b, we can observe this phenomenon. By setting a flow speed of 6.75 mm.s^{-1} , only 6.5% of cells are deflected when they arrive near the electrodes. In contrast, at a much lower flow velocity 5.2 mm.s^{-1} , 88% of cells are successfully deflected.

Hence, as expected, it is possible to influence the cell's trajectory within the cytometer by tuning the value of the critical CM factor with external parameters (flow speed, electric field magnitude and the electrode's geometry). The critical CM factor acts as a tunable threshold to determine the range of $\text{Re}[f_{cm}]$ that a biological cell should have to be repelled and deflected by the electrodes.

IV. COLORECTAL CANCER STEM CELLS SORTING

A. Cell culture and preparation

Thereafter, to validate the sorting efficiency of the proposed UHF-DEP cytometer, the prototype n°2 (Fig. 7) has been used to sort a mixed population of undifferentiated (SW620 DM) and differentiated (SW620 NM) cancer cells with a ratio 50:50. We have used colon cancer cells from the SW620 cell line (isolated from a lymph node with a metastatic origin). Cells have been cultured in different conditions inducing different phenotypes:

- Normal Medium (NM): RPMI 1640 supplemented by 10% SVF, 1% penicillin/streptomycin and 1% sodium pyruvate well appropriated to favor differentiated cancer cells grown
- Define Medium (DM): involving very stringent culture conditions inducing a large CSCs enrichment in selective DMEM/F12 supplemented by 20 ng/mL EGF, 20 ng/mL bFGF, $5 \mu\text{L/mL}$ insulin, 1X N-2 supplement, 100U penicillin and 0.1 mg/mL streptomycin.

Cells from NM and DM culture were then labeled by fluorescence in order to easily evaluate the number of cells deviated by the cytometer from each population. Fluorescent membrane markers used to discriminate cells were PKH26 for SW620 NM cells (red color) PKH67 for SW620 DM cells (green color). Then both labeled cell population were resuspended in an osmotic medium adapted for DEP experiments made from deionized water (ion free), supplemented by sucrose (conductivity: 26 mS/m permittivity: $79\epsilon_0 \text{ F.m}^{-1}$ at 500 MHz and 21°C and $\text{pH}: 7.6$).

Finally, for the experiment, a 50:50 ratio mixed cell population of SW620 NM and DM cells has been injected in the UHF-DEP cytometer.

B. DEP cytometer design °2

The main objective is to isolate CSCs from the cell suspension of interest. Thus, it requires to collect only two subpopulations: one expected to be highly enriched in CSCs and another mainly composed of differentiated cancer cells.

In addition, in the frame of clinical application perspective where the potential occurrence of CSCs within a sample to analyze is limited, a consistent throughput cell sorting capability have to be targeted to be able to analyze a representative number of biological cells in a reasonable time. To do so, we simplified the electrode design of the initial DEP cytometer prototype and optimized the geometry of its microfluidic network (Fig. 7) to propose another design optimized for the target application.

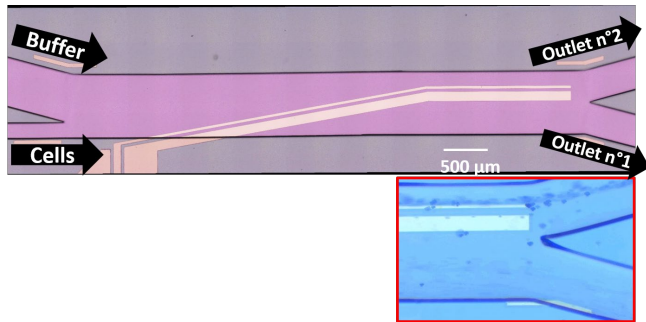


Fig. 7. Second dielectrophoretic cytometer prototype design: focus on the sorting area. The red frame represents the cytometer during a sorting experiment.

The cell focusing area (intersection between the buffer input and the cell input channels), has been re-designed to allow a more accurate control of the cell injection. The number of outlets has been reduced to two in order to collect separately only the two sorted cell sub-populations. The electrode of the UHF-DEP sorting area has been re-designed with a single section owning a much soft slope of 10° in order to be able to operate with higher cell flow rate.

C. Sorting results

As a first proof of concept, the objective of these preliminary experiments was to validate the effect of the sorting frequency f_i on the isolation efficiency of the CSCs subpopulation. At the end of the sorting experiments, cells were collected at the two cytometer outlets and the ratio of green (SW620 DM) and red (SW620 NM) fluorescent cells are evaluated under an optical microscope. As a first estimation, we evaluate the average effective sorting rate during these experiments: at least 25 cells per second. One should note that after flowing through the cytometer, the viability of the cells stayed excellent. Indeed, we observed a mortality rate assessed by trypan blue exclusion assay of only 5%, which remains low. This confirms that the proposed approach is low-invasive and offers the possibility of carrying out additional biological tests on the sorted cells.

The frequency of the DEP signal was tuned in order to evaluate the UHF-DEP cytometer sorting efficiency for the analyzed cell mixture. For this purpose, we selected three frequencies: 480 MHz, 550 MHz and 700MHz for the DEP signal that biased the electrodes at power of 37.7 dBm. Due to unexpected cell adhesion issues occurring below 480MHz, the effective sorting frequencies were finally higher than the predicted theoretical frequency range from Fig. 4 for an optimal sorting. During experiments, we adjusted the pressures applied to the microfluidic network to ensure a

stabilized cell velocity of about $7 \text{ mm}\cdot\text{s}^{-1}$ (flow rate $28.6\mu\text{L}/\text{min}$ for this device).

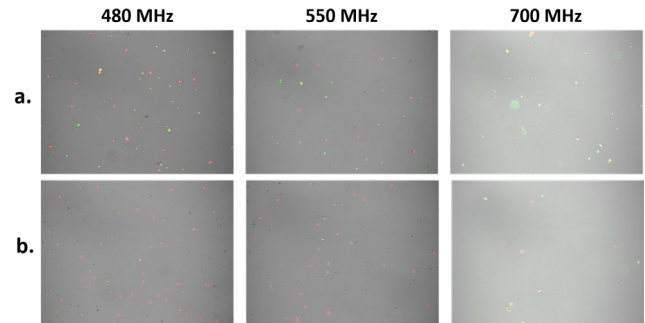


Fig. 8. Optical photographs of collected cells at outlet n°1 (b) and outlet n°2 (a) of the dielectrophoretic cytometer biased at different signal frequencies. Green cells are SW620 DM and red cells are SW620 NM.

As expected, at the two lowest frequencies (480 and 550 MHz), we can see an imbalance between the red and green cells between the two outlets. As one can see on Fig.8, a large majority of cells from outlet n°1 are red whereas cells collected from outlet n°2 are mostly green. Preliminary count of number of cells from each color allowed to establish effective sorting ratios regarding the initial 50% red - 50% green injected mixture. Results are reported in Table III.

TABLE III
PROPORTION OF RED AND GREEN STAINED CELLS COLLECTED AT THE OUTPUT N°2 OF THE UHF-DEP CYTOMETER (FIG. 6) FOR DIFFERENT SIGNAL FREQUENCIES

| Cell type | 480 MHz | 550 MHz | 700 MHz |
|-----------|---------|---------|---------|
| SW620 NM | 30% | 30% | 50% |
| SW620 DM | 70% | 70% | 50% |

We validate that at 480 MHz and 550 MHz, the deviated cells are mostly SW620 DM, cells with a CSC profile while the trajectory of differentiated cells was weakly affected by the repulsive influence of the electrodes. As the frequency of the DEP signal increases (700 MHz), as expected (Fig. 4) more differentiated cancer cells are deflected and the sorting becomes less selective. In fact, at higher f_i , some SW620 NM cells see their $\text{Re}[f_{cm}]$ increases enough in absolute value to exceed the critical CM factor the cytometer. Then, their trajectory can be bent to follow the slope of the electrodes.

These preliminary results are very encouraging and allow to validate the proposed method for label-free CSCs sorting. To the best of our knowledge, it is the first demonstration of successful sorting cancerous stem cells using on UHF-DEP approach. Future investigations will focus on optimizing sorting conditions (DEP signal frequency and magnitude versus cell flow velocity) to reach optimal sorting efficiency, targeting 90-95% sorting ratio.

V. CONCLUSION

In the objective of proposed alternative label-free methods to isolate the sub-population of CSCs, this work has exploited the dielectrophoretic signature specificity of individual biological cells to develop a label-free UHF-DEP cytometer. Operating in a continuous flow and taking advantage of the deflection potential of repulsive forces

generated by an nDEP signal at UHF regime, we have successfully isolated cells presenting an undifferentiated phenotype. The ability of the lab-on-chip device to influence the trajectory of a cell sub-population in a selective way according to their own dielectric properties has been demonstrated with the possibility to modulate the quantity of sorted cells, by adjusting several parameters among which the magnitude of the electric field and the flow velocity. We showed that these parameters allow to adjust the critical CM factor threshold of the cytometer according to the sorting selectivity targeted. Finally, we realized a first sorting experiment using a mixture of colorectal cancer cells made with preliminary labeled CSCs and differentiated cells to evaluate the sorting selectivity and validate the UHF-DEP stem cell discrimination capability.

These first results remain preliminary but very promising for future studies and represent a first proof of concept of the feasibility of sorting and isolating the CSCs subpopulation by a new generation of UHF-DEP cytometer.

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