Direct characterization of circulating DNA in blood plasma using µLAS technology

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***Abstract*—** Circulating cell-free DNA (cfDNA) is a powerful cancer biomarker for establishing targeted therapies or monitoring patients' treatment. However, current cfDNA characterization is severely limited by its low concentration, requiring the extensive use of amplification techniques. Here we report that the µLAS technology allows us to quantitatively characterize the size distribution of purified cfDNA in a few minutes, even when its concentration is as low as 1 pg/µL. Moreover, we show that DNA profiles can be directly measured in blood plasma with a minimal conditioning process to speed up considerably speed up the cfDNA analytical chain.

I. INTRODUCTION

The analysis of cfDNA in body fluids gains in popularity for the management and follow-up of solid tumors. The detection of alterations in cfDNA is likely to guide the administration of cancer drugs as first-line treatments or to fight emerging resistance or relapse (*1*). Various types of DNA alterations have been reported in cfDNA, including point mutations or copies number variation (*2*). In many instances, these alterations were identical to those found in the primary tumor, confirming the relevance of clinical strategies based on monitoring genetic alterations in cfDNA. Additionally, the analysis of cfDNA concentration has often shown an increase in patients' samples correlated with the disease stage (*3*), although the association of cfDNA concentration with an integrity index increases the predictive value of total cfDNA profile (*4*). Here we report the µLAS (µLaboratory for Analysis & Separation) microfluidic technology (*5*) is relevant for cfDNA analysis. We start with a demonstration of the performance of µLAS in the 100-1500 bp size range, which reaches a limit of detection (LOD) of 10 pg/mL and a sizing precision of 3%, We then perform molecular analyses on a small cohort of patients with different types of cancers, and report that the integrity index indeed represents a biomarker for cancer relapse. Finally, we demonstrate that the technology can be operated directly on blood plasma with minimal conditioning protocols.

II. µLAS TECHNOLOGY

A. *µLAS operating principle*

We have recently reported the principle of the µLAS microfluidic technology to perform the operations of DNA concentration and separation simultaneously (*5*). It relies on the manipulation of DNA molecules in a pressure-driven viscoelastic flow in combination with a counter-electrophoretic force. DNA undergoes a viscoelastic force oriented towards the channel walls, the amplitude of which is dependent on its molecular weight (MW). The viscoelastic buffer is composed of 1X Tris-Borate-EDTA (TBE, Sigma) supplemented with 1.3 MDa Polyvinylpyrrolidone (PVP, Sigma) dissolved at 5% in weight, and YOYO-1 (Molecular Probes) at a 1:10 DNA:dye ratio for fluorescence labelling. This technology can also be operated in a linear channel for size separation operations. Concentration is achieved by flowing the DNA solution through a constriction. The funnel shape allows us to modulate the amplitude of the electric and hydrodynamic fields, and hence to generate viscoelastic forces of different amplitudes ahead and past the constriction (Fig. 1A). By setting the pressure drop and voltage appropriately, it is possible to switch from a mode of migration with low forces and molecules predominantly at the centerline of the channel, thus preferentially conveyed by hydrodynamics, to a mode of high forces with molecules stacked to the wall with a dominant contribution of electrophoresis. Overall, DNA velocity is null at the constriction, and DNA molecules accumulate over time because they are transported by hydrodynamics or electrophoresis forward or backward to the funnel, respectively.

This technology relies on conventional pressure and voltage actuation systems. So, it can be operated in microfluidic format using conventional photolithography and plasma etching of silicon (Fig. 1B), as well as on capillary electrophoresis instruments (CE, Fig. 1C). The latter option allows us to compare the performance of µLAS to commercial standards.

B. *Determination of the sizing accuracy & limit of detection*

We first calibrated the µLAS technology with a dual chip design with a reference channel (upper half of Fig. 2) and a calibrated sample with three DNA fragments of 466, 798 and 1512 bp. We fixed the DNA concentration within each band to 80 pg μL^{-1}, which is about three orders of magnitude lower than the typical DNA concentration used for slab gel electrophoresis. We empirically defined the pressure and voltage to be able to visualize all 9 bands of the ladder simultaneously (Fig. 2). This yielded parameters of 6 bar and 82 V, corresponding to a maximum flow velocity and an electric field of ~7 cm s^{-1} and 690 kV m^{-1}, respectively. We set the time of enrichment to 30 s for these experiments. From the resulting fluorescence micrographs, we extracted the intensity profile along the symmetry axis of the two funnels (blue and

red arrows in Fig. 2) and used linear extrapolation between the ladder bands to determine the size of the three target bands. We estimated the size of these three bands to be 456, 788 and 1550 bp, showing that the difference in DNA length between the readout and the nominal size was less than 3%, i.e., an accuracy of ~10 bp for a fragment of 350 bp.

We then evaluated the lower limit of detectable DNA concentration after 5 minutes of concentrating and separating fragments with a reference 100 bp ladder at a total concentration of 1 pg μL^{-1}, or ~100 fg μL^{-1} per band. As expected, we observed a build-up in intensity for all bands of the ladder within 5 minutes (Fig. 1B). Given that current high-sensitivity equipment like the Bioanalyzer or the Fragment Analyzer have a lower limit of detection of about ~5 pg μL^{-1}, according to the manufacturers, the sensitivity of µLAS was 50-fold greater. The LOD can be further improved by increasing the time of concentration to 25 minutes, as carried out with CE. Using serial dilutions of the 100 bp ladder, we focused on the 3 bands of 200, 300, and 1000 bp and deduced respective LODs of 0.07, 0.04, and 0.01 pg μL^{-1} (data not shown). These performances are thus relevant to perform size analysis of highly diluted samples, in particular purified cfDNA samples, as shown in the following paragraph.

III. cfDNA PROFILING

A. *cfDNA profiles*

µLAS technology was then applied to the analysis of cfDNA extracted from blood plasma. cfDNA extraction was carried out starting from 2 mL of fresh plasma using the recommended protocol of Qiagen. We assayed clinical samples from two research teams, each cohort including healthy individuals and patients with Colorectal Cancer (CRC), lung cancer (NLCSC), or melanoma. The cfDNA profiles typically contained a predominant thin peak around 150 bp, a second peak around 300 bp, which is usually smaller and wider, followed or not by a third even smaller and larger peak around 450-500 bp, as well as by various amounts of high MW DNA (greater than 1 kb, upper panel in Fig. 3A). This high MW fraction may come from genomic DNA of leucocytes during pre-analytical stages, or may reflect a physiological phenomenon. This profile was detected for cfDNA samples over a wide range of total DNA concentration spanning 1-500 pg/µL. This typical profile is consistent with previous reports obtained with highly concentrated cfDNA samples analyzed with the bioAnalyzer™ (Agilent) (*6*). Notably however, in our hands (data not shown), when using such state-of-the-art electrophoresis systems, cfDNA profiles could only be assayed for highly concentrated samples with a total DNA concentration larger than ~250 pg/µL.

B. *Validation of the results*

In order to consolidate the quantification of cfDNA performed with µLAS, we compared the total cfDNA concentration to the results of fluorimetry (Qubit™) and digital droplet PCR (dPCR). We first analyzed a set of samples composed of 8 healthy individuals and 14 metastatic colorectal cancers (sample set 1). The correlation between the Qubit™ and µLAS total cfDNA was associated to a Spearman correlation coefficients of 0.8 (left panel in Fig. 3B). The linear regression yielded a proportionality factor between µLAS and Qubit data of 0.82. This value is close to 1, yet a slightly smaller likely because we only quantify DNA molecules in the range 0.1 to 1.6 kb with µLAS in the chosen experimental conditions, whereas the Qubit™ concentration is insensitive to DNA MW. The same sample set was characterized by dPCR with a 60 bp amplicon in the KRAS gene (central panel of Fig. 3B). The Spearman correlation coefficient was 0.8 with a linear regression associated to proportionality factor for µLAS over dPCR concentrations of 1.2, supporting the relevance of our technology to measure total cfDNA concentration. We finally focused on 32 melanoma patients (sample set 2). The correlation plot was quite clear again with a Spearman correlation coefficient 0.89 (right panel of Fig. 4B). Therefore, cfDNA titration by µLAS is consistent with state-of-the-art sensing technologies.

IV. DIRECT cfDNA SENSING IN BLOOD PLASMA

Because the purification of cfDNA is a time-consuming and labor-intensive operation, we set out to establish that profiling could be performed directly in plasma. We therefore optimized a step of protein digestion with proteinase K during 2 hours then centrifuged the sample at 10000 g during 10 minutes and added the visco-elastic solution. Using this approach, we could detect the three main low MW bands characteristic of cfDNA profiles (Fig. 4). Notably, this process is operated in solution containing high amounts of salt, which are typically not adequate to any commercial technology for diluted DNA samples analysis.

V. CONCLUSION

We prove that µLAS is a high sensitivity DNA detection technology that allows cfDNA processing without any steps of molecular amplification.

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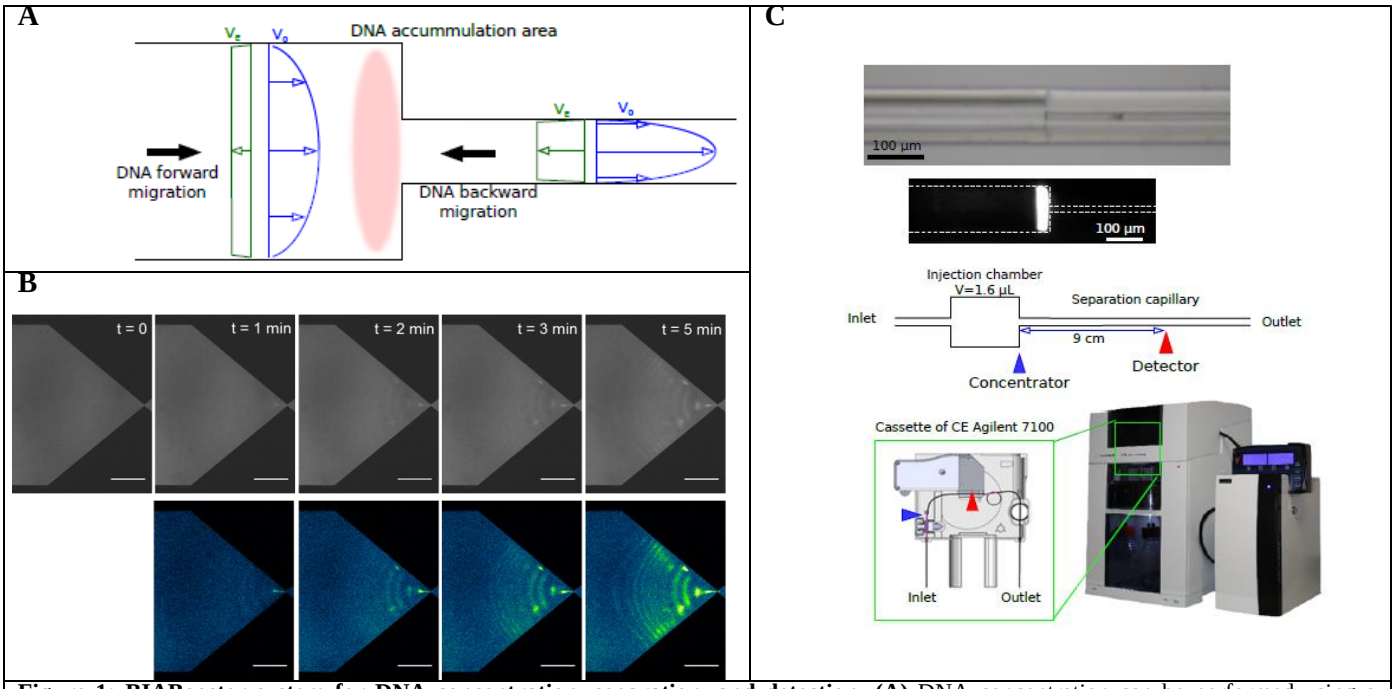

Figure 1: BIABooster system for DNA concentration, separation, and detection. (A) DNA concentration can be performed using a constriction and electro-hydrodynamic actuation. DNA transport is dominated by hydrodynamics (blue arrows) ahead of the constriction and by electrophoresis (green arrows) downstream, so DNA molecules accumulate at the position where their velocity is null. **(B)** Detection of DNA ladder fragments at 100 fg µL^{-1}. The time series in the upper panel shows fluorescence intensity at the constriction using a 100 bp DNA ladder diluted at 1 pg µL^{-1}. Taking the fluorescence micrograph at t=0 s as a reference, the lower panel represents background subtracted intensity profiles, in which the presence of the 9 bands of the ladder appear after 5 minutes. Scale bar = 300 µm. **(C)** Experimental demonstration of the concentration with two hafted capillaries of different inner diameters 100 and 20 µm, as shown in the upper panel. The fluorescence micrograph in the lower panel shows the concentration of 5 kb DNA molecules at the junction. The final device is loaded in an Agilent capillary electrophoresis instrument. Concentration and detection areas are marked with blue and red arrowheads, respectively.

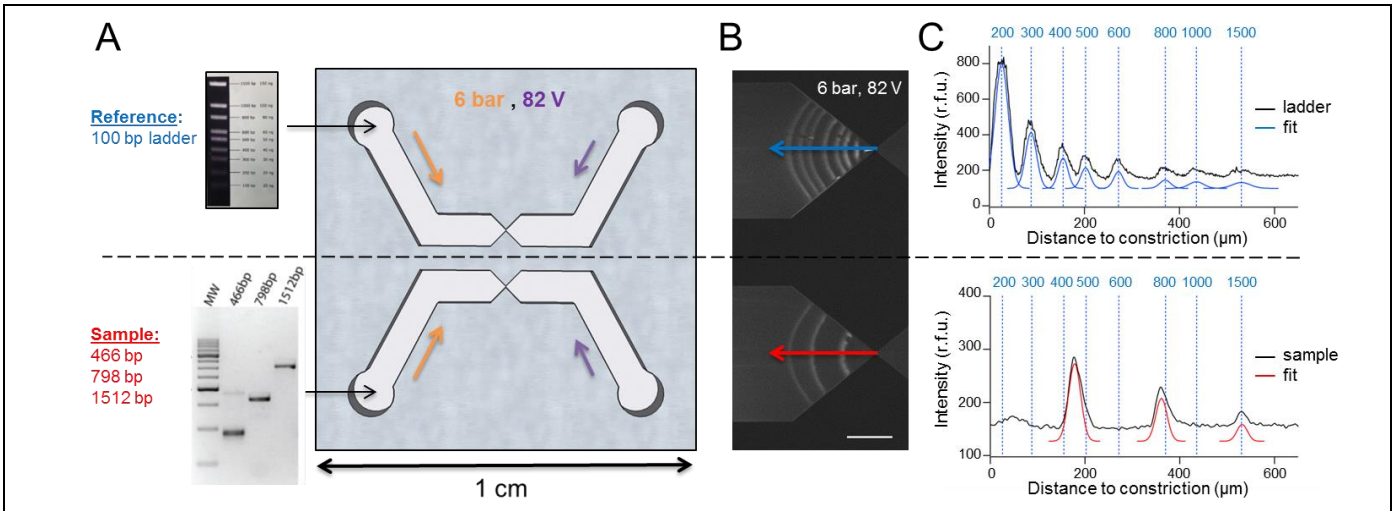

Figure 2: Microfluidic chip for DNA separation and size identification. (A) µLAS chip with two independent channels actuated with the same pressure and voltage. Each channel contains one funnel, in which we operate DNA separation and concentration estimation in line. We used a reference signal with a DNA ladder in the upper channel, and conveyed the analyte in the bottom channel. The 1% TAE agarose gel shown was loaded with 500 ng of DNA/well. **(B)** A fluorescence micrograph shows the two channels after concentration during 30 s using a target sample with three fragments of 466, 798 and 1512 bp at 80 pg µL^{-1}. Scale bar = 300 µm. **(C)** The two plots represent the intensity profile along the two arrows represented in panel (B). The raw data is represented in black and the fits with Gaussian functions by blue and red colors. Based on the position of the center of each Gaussian peak in the ladder (top), we assign the size of the bands by linear interpolation.

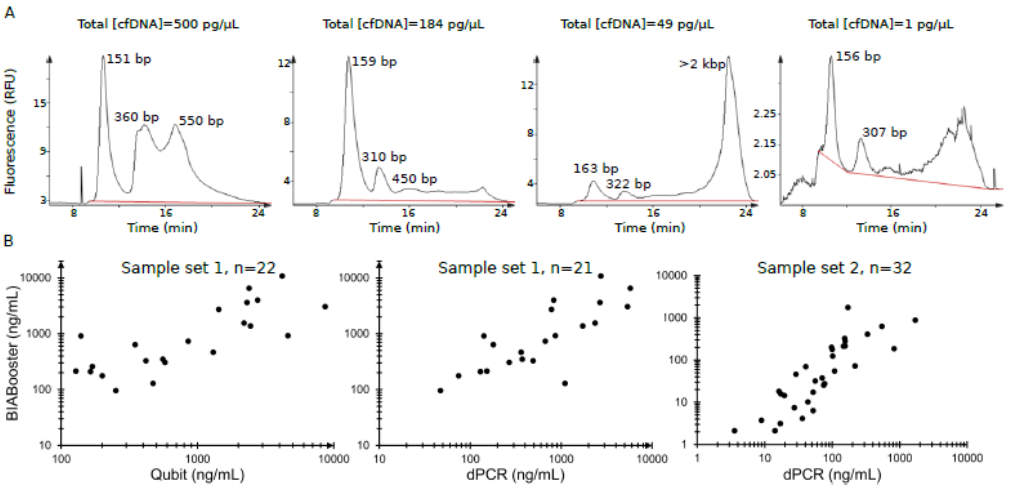

Figure 3: Profiling cfDNA with BIABooster system. (A) typical cfDNA profiles for concentration from 1 to 500 pg/µL (75-1650 bp range). cfDNA samples represented here are from melanoma patients. **(B)** The left plot shows the correlation between cfDNA concentrations of sample set 1 determined by µLAS and fluorimetry. In the middle and right panels, the same comparison is carried out with dPCR for sample set 1 and 2, respectively.

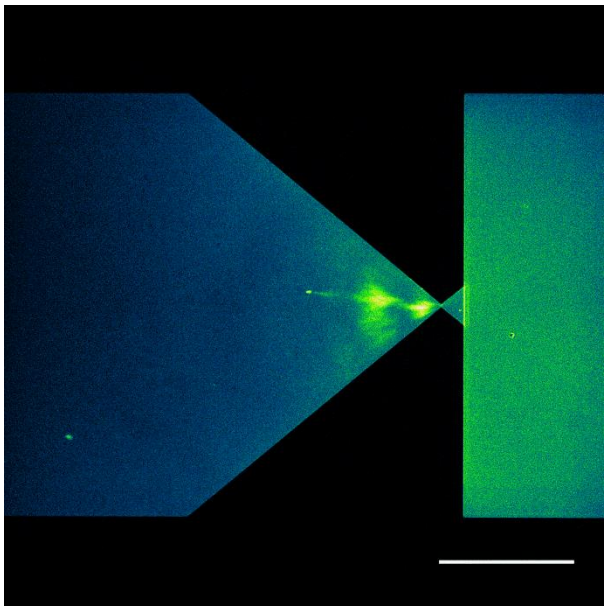

Figure 4: Profiling cfDNA directly from plasma. The fluorescence micrograph shows the cfDNA profile after the processing of plasma with proteinase-K and mixing it with the viscoelastic solution containing 5% PVP.