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# Isolation of Extracellular Vesicles from cell culture and blood through nano-targeted DLD microfluidic device.

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**Running head: Extracellular Vesicles isolation by DLD microfluidic device**

## Abstract

Extracellular Vesicles (EVs) are secreted by cells and found in biological fluids such as blood, with concentration correlated with oncogenic signals, making them attractive biomarkers for liquid biopsy. The current gold-standard method for EVs isolation requires an ultracentrifugation (UC) step among others. The cost and complexity of this technique are forbiddingly high for many researchers, as for clinical implementation. This chapter reports on a simple microfluidic method for EVs isolation, based on a microfluidic size sorting technique named Deterministic Lateral Displacement (DLD). With the design of micrometric DLD-array, we demonstrated the potential of our DLD devices for the isolation of nano-biological objects such as EVs, with main population size distribution consistent with UC technique.

**Key words:** *extracellular vesicles, isolation, Deterministic Lateral Displacement, size sorting, microfluidic*

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## 1. Introduction

Extracellular Vesicles (EVs) are lipid nanoparticles secreted by cells for communication purposes that circulate in biological fluids as blood [1]. They convey information from a parental cell to another cell thanks to proteins displayed on their surface and their DNA/RNA content. As their abundance and the expression of specific surface proteins have been shown to vary in correlation with specific pathologies, mainly cancers, they are considered as promising biomarkers in the field of liquid biopsies[2]. EVs isolation

is however a technological challenge. It is currently mostly addressed using differential centrifugation, as described by Théry *et al.* in 2006 [3]. However, this method involves the use of an ultracentrifuge apparatus, which turns out to be labor intensive, time and cost consuming, and requires significant sample volumes to ensure sufficient reproducibility. Because of the significant number of limitations due to differential centrifugation, the International Society of Extracellular Vesicles (ISEV) has encouraged the development of new methods for EVs isolation in 2018 [4], fostering samples and measurement protocols standardization, sufficient reproducibility, speed, and simplicity for operators.

Microfluidic-based techniques may address such issues by enabling the analysis of a low biological sample volume and in a simpler manner than centrifugation-based approaches. Deterministic Lateral Displacement (DLD) is a size sorting microfluidic technique based on a slightly disoriented pillar array [5]. DLD network geometry defines a critical diameter ( $D_c$ ) according to which particles of larger sizes are deviated according to the pillar array orientation while smaller ones are not. During the last decade, this technique has been successfully applied to the sorting of micrometric biological objects: blood cells [6], circulating tumor cells [7] and bacteria [8] among others. As recently reported by Hochstetter *et al.*, nowadays the main challenge of DLD is the sorting of nanoparticles [9]. Previous works have already demonstrated the ability to sort nanovesicles using nanoscale DLD arrays but required restrictive preparation steps [10,11]. Practically, EVs are isolated and concentrated by ultracentrifugation before being sorted by nanoscale DLD. However, there is still a lack of simple operation of DLD onto EVs samples. The approach we describe in this chapter aims at sorting nanometric particles using micrometer-sized pillar array (Figure 1), rather than nanometer-sized structures which are way more challenging to fabricate than our initial micrometric DLD sorting system [12]. Micrometric pillar arrays enable to decrease both the fluidic resistance and the overall processing time, and in the meantime significantly increase the flow rate through the device by comparison to nanometric structures. This DLD approach allows our system to be generic for a large range of nanometric biological objects. The proposed method is based on the association of (i) two low-centrifugation steps, meant to guarantee the absence of large particles / cell debris that could generate clogging in the DLD channel, with

(ii) the sorting processing through DLD system. In this chapter, we propose a detailed practical procedure to successfully carry out EVs sorting (Figure 2), either from culture media or blood, with the DLD technique. It will cover the description of preliminary steps, such as biological sample preparation and DLD device fabrication, as well as the fluidic implementation of EVs sorting through DLD and characterization of the sorted samples through Nano-Tracking Analysis (NTA). A comparison with UC classical isolation protocol will also be provided, showing similar isolation of EVs subpopulations with a comparable precision, but processing lower sample volumes, which is an asset for biological samples.

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## 2. Materials

### 2.1. Biological samples and reagents

1. THP-1 cells from ATCC
2. THP-1 cell culture medium composed of Rosewell Park Memorial Institute Medium (RPMI) 1640 Glutamax media supplemented with 10% Foetal Bovine Serum FBS (v:v) and 1% PenStrep (10,000 U/mL and 10 mg/mL Penicillin and Streptomycin respectively) (v:v)
3. EVs-free RPMI 1640 composed of RPMI 1640 Glutamax media supplemented with 20% Foetal Bovine Serum FBS (v:v) and 1% PenStrep (v:v) and centrifuged at 100,000g for 18 h at 4°C. Supernatant is collected, diluted (dilution factor = 2) in RPMI 1640 Glutamax media supplemented with 1% PenStrep (10,000 U/mL and 10 mg/mL Penicillin and Streptomycin respectively) (v:v) and is stored at -20°C
4. MCF-7 cells from ATCC
5. MCF-7 cell culture medium composed of Modified Eagle Medium (MEM) Glutamax media supplemented with 10% FBS (v:v) and 1% PenStrep (v:v)
6. Preparation of EVs-free MEM composed of Modified Eagle Medium (MEM) Glutamax media supplemented with 10% FBS (v:v) and 1% PenStrep (v:v) and centrifuged at 100,000g for 18 h at

4°C. Supernatant is collected, diluted (dilution factor = 2) in MEM Glutamax media supplemented with 1% PenStrep (v:v) and is stored at -20°C

7. Blood samples: 5 × 10 mL of blood (heparin as anticoagulant). Blood donor volunteers must be informed and give their written consent for the scientific use of their blood, according to procedures defined by national Health and Bio-ethics rules.
8. PBS filtered onto 0.22 μm

## **2.2. Nano-targeted DLD microfluidic device**

### **2.2.1. DLD silicon device**

The DLD critical diameter  $D_c$  has to be adjusted according to the size range of the population of interest. For EVs from THP-1 and MCF-7 cells as well as for EVs from plasma, the size range is 50 nm – 300 nm. In this chapter, we will then focus on a DLD channel with a  $D_c$  of 350 nm, keeping in mind that it should be adjusted depending on the size of the targeted population.

Reaching a submicrometric  $D_c$ , while targeting deep channels (i.e., high pillar height to diameter aspect ratio) to optimize the sample flow rate, requires the use of silicon technologies for the fabrication of DLD channels. In addition to the reproducibility and reliability brought by these fabrication techniques, it is possible to address micrometric and submicrometric scales and high aspect ratio structures. The main features we are using are described in Table 1 and the main fabrication steps are illustrated on Figure 3.

A DLD chip is composed of (i) a silicon substrate including the DLD fluidic channel and the fluidic inlets/outlets and (ii) a glass cover closing the fluidic system. These 200 mm substrates are assembled at wafer scale in cleanroom with an anodic bonding process. Preliminary to this step, the DLD pillar network is patterned in the silicon substrate through a DRIE process (Deep Reactive Ion Etching), leading to pillars aspect Ratio of 15. After anodic bonding, the fabrication process is completed with the patterning of fluidic inlets/outlets (DRIE process) in the silicon substrate. 200 mm wafers are then diced into chips.

These chips can however not be directly connected to a fluidic sample nor an actuation system (syringe pumps, pressure controllers), and they require a fluidic packaging as an interface, referred to as a “microfluidic cartridge”.

### **2.2.2. Microfluidic cartridge in Cyclic Olefin Copolymer (COC)**

This microfluidic cartridge consists in a polymer substrate on which the silicon chip is laid and a screwed metallic frame, featuring holes for tubing insertion. Between these two parts, ferrules and homemade silicon gaskets ensure fluidic sealing (Figure 4).

The polymer substrate is made from two thermally sealed COC polymer layers and houses micromachined fluidic channels (200  $\mu\text{m}$  in diameter). The screwed metallic frame is meant to ensure a good sealing between the tubing and the polymer layer on one hand, and between the polymer layer and the silicon chip on the other hand, while a pressure of a few bars is applied on the fluidic sample at the inlet.

### **2.2.3. Device assembly:**

1. Aluminum frame and screws: The metallic frame (8.5 mm\*5.4 mm) is made from aluminum sheet and is in-house manufactured. Its center is hollowed (window of 5.5 mm\*1.8 mm) for optical monitoring of the DLD channel during fluidic filling. It includes holes for screw, as well as for tubing connection with the microfluidic cartridge. Sealing between tubing and microfluidic cartridge is ensured by ferrules.
2. Tubing: The fluidic tubing used is PTFE 1/16 inch  $\times$  0.8 mm. About 15 cm length are used of each inlet/outlet.
3. Inlet and outlets ferrules: The ferrules used for inlet and outlets rings are compatible with 1/16 inch outside diameter tubing.
4. Silicon gaskets: Silicon gaskets are molded using silicon Nusil MED-6010, with a mold corresponding to the fluidic inlets/outlets of the silicon chip (holes of 500  $\mu\text{m}$  in diameter).
5. Greiner<sup>®</sup> 15 mL centrifuge tubes, used as containers for sample injection into the microfluidic system inlet

6. Adapted cap for centrifuge tube: The centrifuge tube is closed with a Teflon cap, enabling both a pneumatic interface (for the pressurization of the sample) and a fluidic interface (for the sample injection towards DLD). This cap is homemade and considers the pneumatic and fluidic connectors diameters for the holes sizing (Figure 5).
7. 2 mL Eppendorf® tubes, used as containers for the collection of the sorted sample at the microfluidic system outlets

### **2.3. Equipment**

1. Low-speed centrifuge (up to 2,500 g) for 50 mL Falcon® tubes
2. High-speed centrifuge (Beckmann, Optima L-100K)
3. Direct Microscope equipped with an objective turret (2.5 x and 10 x) and a sensitive CCD camera (Hamamatsu Orca Flash 4.0LT+) controlled by computer through software (HCImageLive 4.4.2)
4. Fluigent pressure controller MFCS™-EZ controlled by computer through software Maesflo 3.4.0
5. Nanosight NS300, Malvern Panalytical controlled by computer through software NTA 3.4 and associated syringe pump (Nanosight syringe pump, Malvern Panalytical), controlled by computer through software NTA 3.4

### **2.4. Consumables**

1. Luer filter Amicon® Ultra-15K 100K
2. Luer filter 1.2 µm
3. Filter 0.22 µm
4. 50 mL Falcon® tubes
5. T75 (75 cm<sup>2</sup>) flasks

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## **3. Methods**

### **3.1. Preparation of biological samples**

#### **3.1.1. THP-1 cell culture**

1. THP-1 cells are cultured in T75 (75 cm<sup>2</sup>) flasks (two flasks per run, 2 × 12 mL) in THP-1 cell culture medium. Culture media are changed every 48-72 h and cells are passed at 80% of confluence in maximum (130,000 cells/cm<sup>2</sup> or 800,000 cells/mL).
2. 48 h before EVs isolation, culture medium is removed by pipetting and EVs-free RPMI 1640 + 10% FBS + 1% PenStrep fresh medium is added.
3. The content of two T75 flasks of THP-1 cells (2 × 12 mL) is collected, and supernatant is centrifuged at 300 g for 10 min at room temperature in 15 mL centrifuge tubes.
4. Cells are pelleted and the resulting supernatant is collected, transferred into two 15 mL centrifuge tubes, and centrifuged at 2,000 g for 10 min at room temperature. Dead cells and large cell debris are pelleted, and the supernatant is collected (2 × 10 mL) and concentrated onto Amicon<sup>®</sup> Ultra-15K 100K filter (concentration factor = 5) and filtered onto a luer connected 1.2 µm filter on a 10 mL syringe. Approximate collected volume is 2 mL and is placed into a Greiner<sup>®</sup> 15 mL tube.
5. The biological fluid, placed into Greiner<sup>®</sup> tube, is ready to be injected, see Method (3.2). Biological samples are preferably used up to 6 h after preparation at room temperature.

### **3.1.2. MCF-7 cell culture**

1. MCF-7 cells are cultured in T75 flasks (two flasks per run, 2 × 12mL) in MCF-7 cell culture medium. Culture media are changed every 48-72h and cells are passed at 80% of confluence in maximum (13,000 cells/cm<sup>2</sup> or 80,000 cells/mL).
2. 48 h before EVs isolation, culture medium is removed by pipetting and EVs-free MEM + 10% FBS +1% PenStrep fresh medium is added.
3. The content of two T75 flasks of MCF-7 cells is collected, and supernatant is centrifuged at 300 g for 10 min at room temperature in two 15 mL centrifuge tubes.
4. Cells are pelleted and the resulting supernatant collected, transferred into two 15 mL centrifuge tubes, and centrifuged at 2,000 g for 10 min at room temperature. Dead cells and large cell debris



are pelleted, and the supernatant is collected ( $2 \times 10$  mL) and concentrated onto Amicon<sup>®</sup> Ultra-15K 100K filter (concentration factor = 5) and filtered onto a luer connected 1.2  $\mu$ m filter on a 10 mL syringe. Approximate collected volume is 2 mL and is placed into a Greiner<sup>®</sup> 15 mL tube.

5. The biological fluid, placed into Greiner<sup>®</sup> tube, is ready to be injected, see Method (3.2). Biological samples are preferably used up to 6 h after preparation at room temperature.

### **3.1.3. Preparing human plasma from blood**

1. 50 mL of blood samples are pooled together and poured in the same 50 mL centrifuge tube. The sample is centrifuged for 15 min at 2,500 g at room temperature to remove blood cells (red blood cells, white blood cells and platelets).
2. The supernatant is collected and centrifuged a second time, for 15 min at 2,500 g at room temperature to remove residual blood cells.
3. The supernatant (plasma) is then slowly collected by pipetting and avoiding the collection of any precipitated material (about 40-50% of the total volume).
4. Once the plasma is collected, it is diluted at 1:10 in PBS.
5. The biological sample is filtered onto a luer connected 1.2  $\mu$ m filter on a 10 mL syringe and placed into a Greiner<sup>®</sup> 15 mL tube (EVs are concentrated at about  $10^{10}$ /mL).
6. The biological fluid, placed into the Greiner<sup>®</sup> tube, is ready to be injected, see Method (3.2). Biological samples are preferably used up to 6 h after preparation at room temperature.

### **3.2. Implementation of DLD microfluidic device and EVs sorting (Figure 2)**

1. A DLD silicon chip is taken off from storage box.
2. Silicon gaskets are precisely aligned with the silicon chip inlets and outlets (see Note 1).

3. The metallic frame is equipped with appropriate inlet and outlets tubing associated with ferrules (Figure 2, picture 1).
4. DLD chip is sandwiched between the metallic frame and the plastic microfluidic cartridge, and the system is manually screwed (see Note 2) (Figure 2, picture 2).
5. The system is set under the microscope associated to the sensitive camera (Figure 2, picture 3).
6. The previously prepared biological sample (see Methods 1.1-1.3) is added to a specific Greiner<sup>®</sup> 15 mL centrifuge tube and connected to the DLD system with the inlet tubing, which is adjusted through the centrifuge tube via the adapted cap to ensure the sealing (Figure 2, picture 4).
7. DLD outlets are connected to Eppendorf<sup>®</sup> tubes (through the outlet tubings) for sample collection after sorting. Considering a DLD channel with a critical diameter of 350nm, EVs are expected to flow without being deviated and to be collected in the non-deviated outlets. Larger particles are expected to be separated from EVs while being deviated by the pillar array up to the deviated outlet.
8. The biological sample is injected into the DLD device by increasing the pression up to 2 bars with the pressure controller, for about 3 minutes (filling step) (see Notes 3 and 4).
9. The filling progress into the channel is monitored with the microscope and camera.
10. When the DLD system is properly filled with the sample, injection pression is raised up to 4 bars and the sorting step begins. It lasts approximately 6 h for a 2 mL sample (flowrate in the order of 350  $\mu$ L/h).
11. Sorted samples are collected in Eppendorf<sup>®</sup> tubes at DLD outlets.

### **3.3. Size characterization of Extracellular Vesicles samples using NTA**

EVs collected in non-deviated outlets are characterized in size by NTA (see Note 5).

#### **3.3.1. Recommendations before analysis**

An ideal volume of 1mL and an ideal concentration of about 50 particles/image (acceptable if higher than 10-15 particles/image) and corresponding to a sample concentration of  $10^7$  to  $10^9$  particles/mL are usually

recommended for a relevant analysis. Isolated EVs may thus have to be diluted in filtered PBS. UC-isolated EVs are typically diluted with a factor 1,000 before NTA analysis while DLD-isolated EVs are usually not diluted.

### **3.3.2. Analysis**

1. The NTA associated syringe pump is set to have a flow rate of 50 units.
2. The focus and camera level are adjusted according to particles type (camera level is usually between 14 and 16 for EVs).
3. The number of particles per frame is in the order of 50 otherwise the sample dilution has to be adjusted.
4. Three captures of 60 to 90 sec are usually performed.
5. Data are then processed, and the detection threshold is chosen. This detection threshold corresponds to the number of pixels to be considered as a particle (usually set at 7-8 in our study).

At the end of this protocol, EVs size profile allows EVs sample characterization and informs about the efficiency of EVs isolation through DLD device. EVs subpopulations can be compared for different biological samples or for different isolation protocols. In our case, we can note that the NTA size profile of EVs isolated by DLD device extends from 50 to 300 nm (Figure 6), and that the major EVs subpopulation around 100 nm is very similar for both DLD and UC approaches. However, lower sample volumes can be processed using the DLD microfluidic system, which is an asset for biological samples. Samples volumes and concentrations used in both approaches are summarized in Table 2.

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## **4. Notes**

1. Sealing tips with silicon gaskets: As mentioned above, silicon gaskets are fabricated at CEA-Leti using silicon Nusil MED-6010. If these types of gaskets are not available, O-ring gaskets can be adapted to each inlet/outlets of the DLD silicon chip.

2. Tips for the screwing of DLD device: a fine tightening of the screws of the microfluidic device is necessary to ensure a good seal while avoiding breaking the DLD silicon component which is relatively fragile. It is recommended to screw the screws crosswise and to check the good crushing of the gaskets.
3. Avoiding bubbles in DLD: the tubing is gently placed in the biological sample and the injection pressure is fixed at 2 bars to avoid the appearance of bubbles and to ensure the good filling of the microfluidic channel.
4. Avoiding clogging in DLD: in microfluidics, clogging is a crucial issue. In DLD and particularly in nano-targeted DLD application, clogging must be avoided for the longest time possible. We propose to prevent it by following the recommendations established in this paper (i.e., concentration of particles in biological samples of about  $10^8$  part/mL and filtration of the buffer). We evaluated that pre-conditioning is not required and the sample is directly injected into the DLD device.
5. Ensuring a good NTA characterization: NTA analysis is optimal when the number of particles per image is about 50 so that each particle can scatter the light relative to itself independently. Since EVs are biological particles, their refractive index is relatively low implying a camera level setting between 14 and 16.

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**Figure Captions:**

Figure 1: Pictures of nano-targeted DLD device: a) different components of the DLD device, b) assembled DLD device.

Figure 2: Scheme of EVs sorting steps with DLD.

Figure 3: Global DLD chip process flow.

Figure 4: Fluidic packaging of nano-targeted DLD microfluidic device: a) Schematic of the cross-sectional view of the nano-targeted DLD device, b) Assembled nano-targeted DLD device on the front and back side.

Figure 5: Picture of DLD microfluidic device in the experimental set-up.

Figure 6: Comparison of size and concentration profiles for EVs from THP-1 cells isolated either by DLD ( $D_c=350\text{nm}$ ) (blue curve) or by the standard UC method (purple curve).

## **Table Captions**

Table 1: Main features of DLD silicon chip  $D_c = 350\text{nm}$ .

Table 2: Summary of cell media needed and collected EVs by UC and DLD isolation methods.



## Tables

Table 1:

DLD $D_c = 350\text{nm}$	
Pillar diameter ( $D_p$ )	2,5 $\mu\text{m}$
Interpillar gap (G)	2,5 $\mu\text{m}$
Period (N)	70
Channel depth (H)	37,5 $\mu\text{m}$
Array length (L)	44 mm
Array width (W)	575 $\mu\text{m}$

Table 2:

	Starting Cells				Collected EVs			
	Biological sample preparation		Injected volume (mL)		UC		DLD	
	Concentration (cells/mL)	Volume (mL)	UC	DLD	Concentration (EVs/mL)	Volume ( $\mu\text{L}$ )	Concentration (EVs/mL)	Volume (mL)
<b>THP-1 cell line</b>	800,000	24	24	2	$7 \times 10^{10}$	500	$3 \times 10^9$	2
<b>MCF-7 cell line</b>	80,000	24	24	2	$2 \times 10^{11}$	500	$6 \times 10^9$	2
<b>Blood</b>	5,000,000	50	25	2	$2 \times 10^{11}$	500	$7 \times 10^9$	2

# 6. Figures

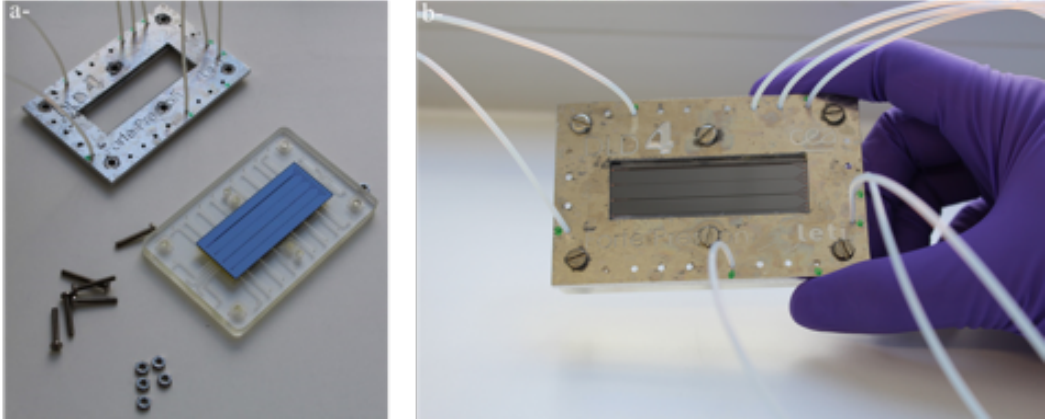


Figure 1

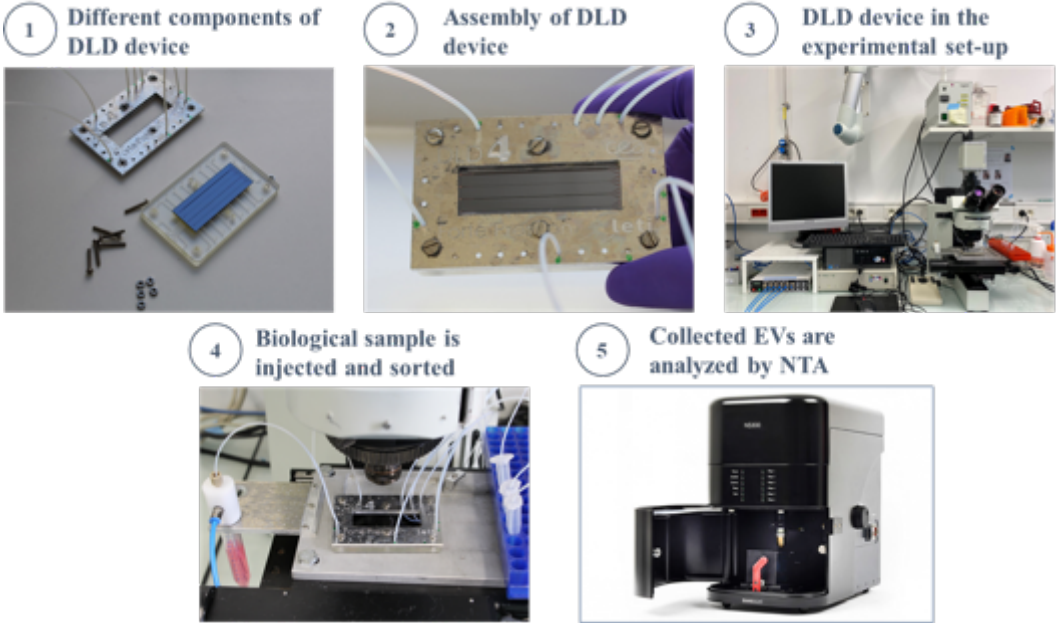


Figure 2

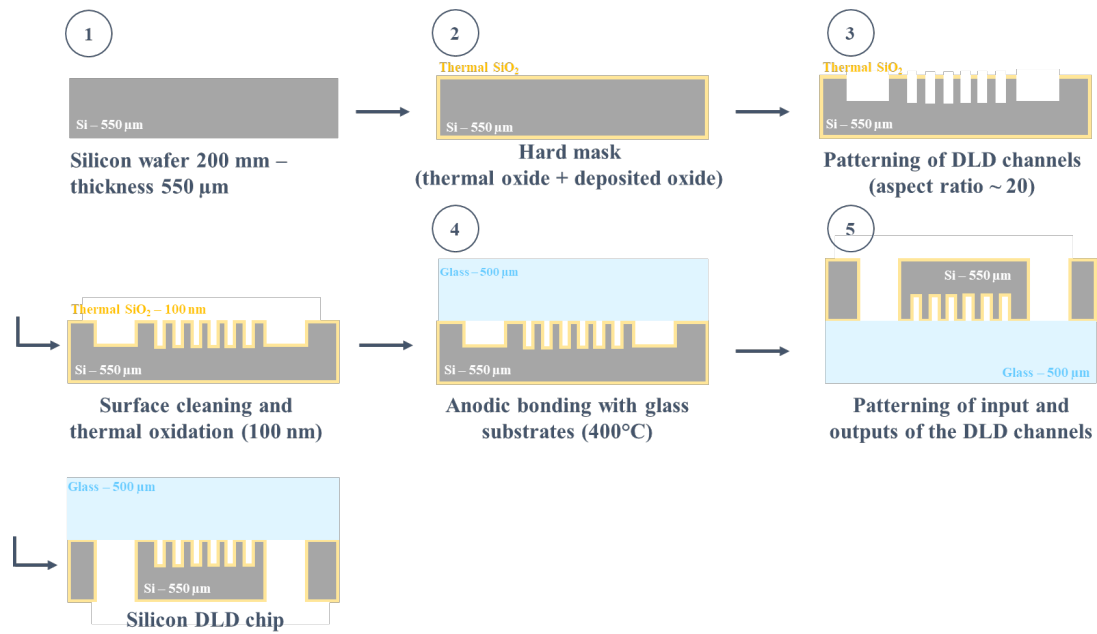


Figure 3

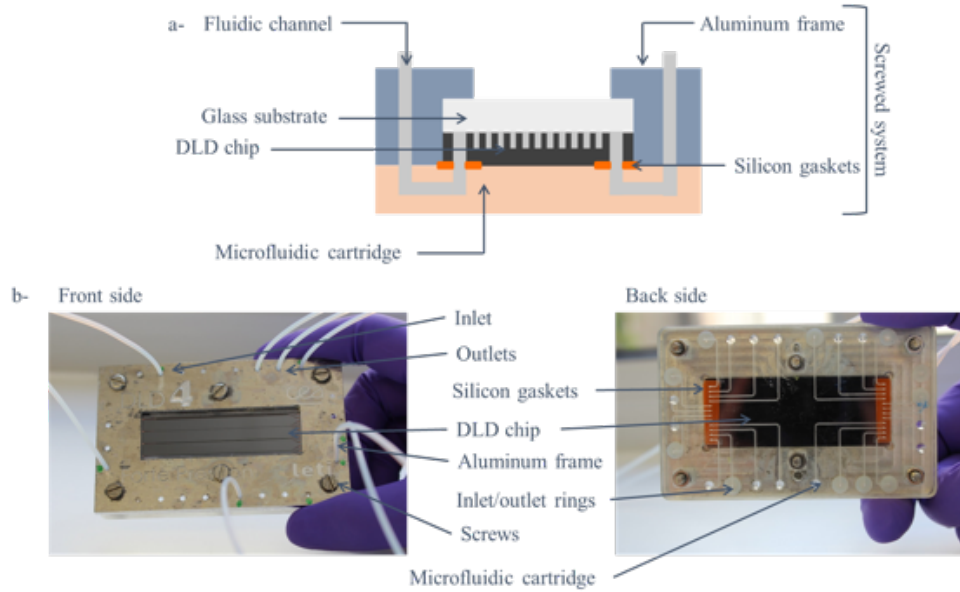


Figure 4

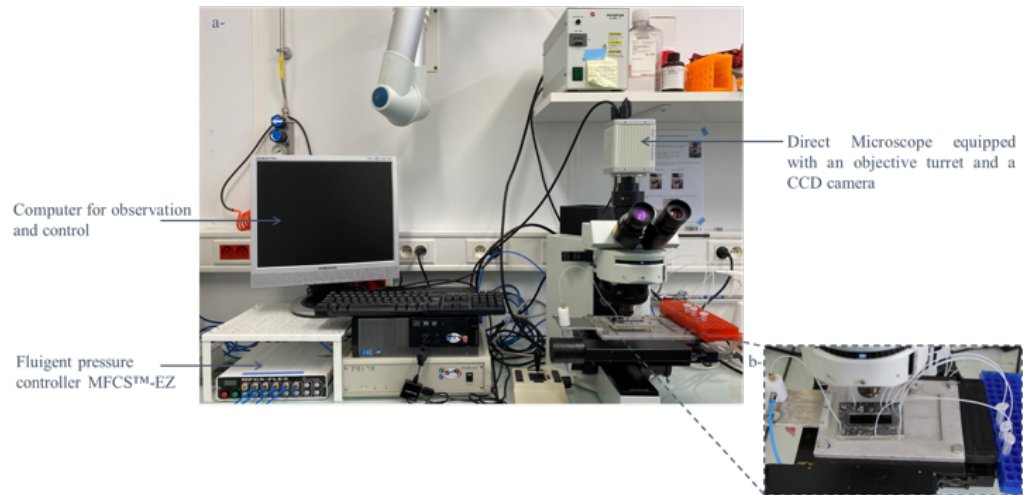


Figure 5

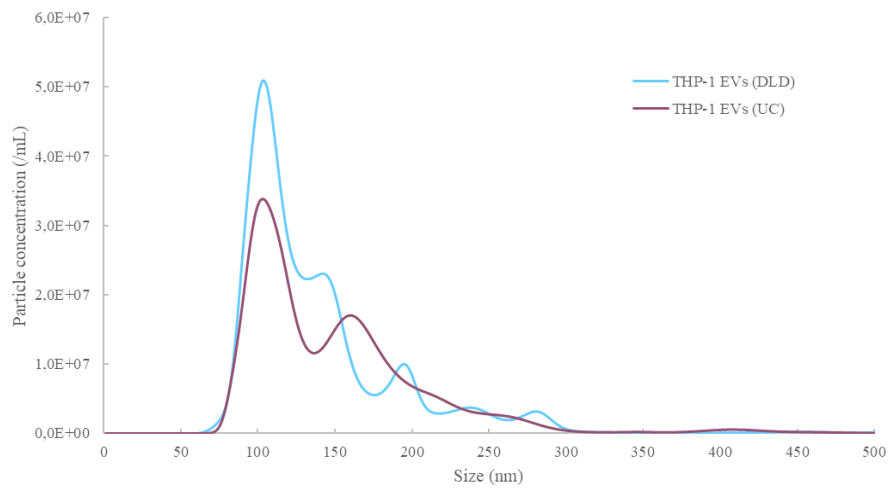


Figure 6