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# 3D Microfluidic Platforms for Extracellular Vesicle Isolation, Characterization, Downstream Analysis and In Vitro Treatment V.2

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**We use this protocol and it's working**

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## Disclaimer

This chapter is for publication in an upcoming volume of “Methods in Molecular Biology”

## Abstract

**Abstract**

Extracellular vesicles (EVs) are small membranous vesicles secreted by most cell types that play important roles in cell-to-cell communication. Despite the tremendous work in the field, there are still challenges to studying organ-crosstalk mediated by EVs in complex organisms. 3D microfluidic platforms offer promising and novel platforms for EV research to overcome the challenges in complex biological settings. In this chapter, we provide a protocol for the use of 3D systems to study the effect but also to isolate EVs from these physiological relevant complex settings.

## Image Attribution

**Figure 1. Successful dosing of extracellular vesicles on cells.** Maleimide-stained EVs from LOC effluent were visualized after 72 h perfusion period using fluorescence microscopy. Representative images of the fluorescently labelled EVs (red).

**Figure 2. Characterization of EVs from LOC outlet effluent.** We were able to characterize the liver-on-chip (LOC) effluent derived extracellular vesicles (EVs) following the MISEV guidelines (**Fig. 2**). **(A)** For particle size distribution we performed Microfluidic Resistive Pulse Sensing (MRPS). **(B)** We performed immunoblot with canonical EV specific markers (CD81 and TSG101) along with hepatocytes specific EV markers (CYP2E1 and ASGR1). **(C)** Finally, we visualized the typical cup shaped EV structure by transmission electron microscopy (TEM) (Green arrows indicate immuno-labelled CYP2E1 on EVs; scale bar used = 200 nm).

**Figure 3. Representative Bioanalyzer traces of RNA and cDNA libraries from renal proximal tubule epithelial cells (RPTECs) and renal glomerular microvascular endothelial cells (RMVECs) following treatment with normal placental extracellular vesicles in the kidney-chip model.** RPTECs (A and C) and RMVECs (B and D) were either untreated (media-only controls, blue traces) or treated with normal placental EVs (red traces) at a dose of 1 x $10^{10}$ EVs/mL for 72 h in the kidney-chip model prior to RNA extraction (A and B) and cDNA library preparation (C and D) for bulk RNA-Seq. cDNA libraries were diluted appropriately to be within the detection range of the High Sensitivity DNA Kit chip (Agilent).

## Materials

**Organ-on-a-chip using Emulate chip:**

1. Liver-on-chip (LOC) bio kit which includes PDMS Chips (S1), Pods, (Emulate. Inc), Hepatocytes, Kupffer ells, Stellate cells, and liver endothelial cells
2. Kidney-on-chip (KOC) bio kit which includes PDMS chips, Pods, renal proximal tubule epithelial cells (RPTECs), and renal glomerular microvascular endothelial cells (RMVECs)
3. Zoë (Emulate, inc.)
4. ORB hub module (Emulate, inc.)
5. 1.5 ml Eppendorf tubes
6. Pipettes and multichannel pipettes
7. Weighing scale
8. Automated Size Exclusion Chromatography (Automated SEC) (see chapter: “Isolation and Characterization of Tissue and Cell-Derived Extracellular Vesicles and Non-Vesicular Extracellular Particles” for detailed method)

**Labelling of extracellular vesicles and *in vitro* treatment**

1. Maleimide-dye (ThermoFisher Scientific, cat. no. R6029)
2. DMSO
3. 1X PBS
4. Glutathione (Sigma cat.no. 70-18-8)
5. Aluminium foil
6. qEV single column (Izon, 70nm, gen 2)
7. Automated SEC
8. Ultracentrifugal filter 10KDa MWCO (Amicon, cat.no.UFC 810024)
9. 1.5 mL Eppendorf tubes
10. Pipettes

**Gas chromatography-mass spectrometry (GC-MS)-based metabolomics analysis**

1. Saline Solution: 0.9% NaCl in $H_2O$ (weight/volume) (sterile, stored at 4°C).
2. Media Extraction Mix: 80:20 MtOH:$H_2O$ with 500mM isotypically labelled amino acid standards (Cambridge Isotope Labs MSK-A2-1.2)
3. Optima LCMS Grade Methanol and $H_2O$ mixed in clean glass wear, (stored at -20°C) (***see,* Note below**).

> **Note**
>
> Always use detergent-free glassware.

4. $H_2O$, Optima LCMS Grade (stored at 4°C)
5. HPLC grade Chloroform (stored at -20°C)
6. Vortex
7. Centrifuge machine

8. Speed vac
9. 1.5 mL Eppendorf tubes
10. Pipettes
11. Glassware

**Bulk RNA-Seq of kidney-chip cells treated with extracellular vesicles**

1. PureLink RNA Mini Kit (ThermoFisher Scientific, cat no. 12183018A)
2. 2-Mercaptoethanol to add to lysis buffer in PureLink RNA Mini Kit
3. Ambion DNA-free$^{M}$ DNA Removal Kit (ThermoFisher Scientfic, cat. No AM1906)
4. KAPA RNA HyperPrep Kit with RiboErase (HMR) (Roche, cat. no. 08098140702)
5. KAPA Unique Dula-Indexed ADAPTER Kit (15 µM) (Roche, cat. No 08861919702
6. Agilent RNA 6000 Nano Kit (cat. no. 5067-1511)
7. Agilent High Sensitivity DNA Kit (cat. no. 5067-4626)
8. Bioanalyzer 2100 (Agilent Technologies)
9. Nanodrop spectrophotometer
10. Pipettes
11. BIOTIX$^{TM}$ Rainin LTS filter tips (sterile)
12. Benchtop centrifuge
13. Bioruptor UCD-200TM ultrasonic bath (Tosho Denki)
14. Molecular grade water

1 **Organ-on-a-chip**

1.1
- Liver-on-chip:
  Follow Manufacturer's protocol for seeding chip with hepatocytes, Kupffer cells, Stellate cells, and liver endothelial cells (Emulate, inc.).
- Kidney-on-chip:
  Follow Manufacturer's protocol for seeding chip with RPTECs and RMVECs (Emulate, inc.).

2 **Extracellular Vesicles for *in vitro* treatment and imaging**

For treating the cells with EVs, two procedures should be run independently, see below for details:

2.1 *Labelling EVs with maleimide-dye to confirm EVs uptake by cells*

1. Resuspend a maleimide-dye aliquot (20 nmol) in 5 µL DMSO.
2. Label 150 µL of EVs with 8 nmol of dye (2 µL) (***see,* Note below**).

> **Note**
>
> Calculate the amount of dye according to the volume of EVs needed.

3. Cover the tubes with aluminium foil and incubate for 1 h RT on rotator
4. Quench for 10 minutes with glutathione (GSH).
5. To remove excess dye pre incubate the qEV single column with 4 mL 1XPBS at room temperature.
6. Add 150 µL of dye labeled EV solution
7. Once all of the EV solution is through the frit, add 850 µL PBS (void volume is 1 mL).
8. After the void volume as passed through the column, add 600 µL of PBS and begin collecting into a fresh 100 kDa concentrator.
9. Concentrate resulting 600 µL of labeled EV containing fractions down to less than 150 uL for treating the cells.

2.2 *Treatment of cells with extracellular vesicles*

(Before treating cells with EVs ***see,* Note below**).

Note

Seed cells so that they are confluent at the time of treatment e.g. 100,000-150,000 cells/well into 96 well plates.

1. Measure the concentration of the EVs using Microfluidic Resistive Pulse Sensing (MRPS: Spectradyne's nCS1) (***see*, Note below**).

Note

Pay attention to the cartridge selection, we use C400 cartridge which covers a size range from 65nm to 400nm, this size range is ideal for small EV analysis.

2. Adjust the volume of the isolated EV so that the calculated exposure of EVs/ cell should within 6,000-7000 EVs/cell during the treatment period.

CITATION

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LINK

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3. Combine solated EVs with the cell-specific media to reach the desired number of EVs/cells.
4. Treat the cells with EVs for 72 h (***see*, Note below**).

Note

While treating the cells with EVs always include a negative control group by treating the cells with same volume of 1X PBS combined with respective media.

5. After 72 h of treatment check the cells under fluorescence microscope. We were able to detect the uptake of fluorescent EVs in the cells, which suggests an

effective delivery of EVs (**Figure 1**).

Expected result

Figure 1.

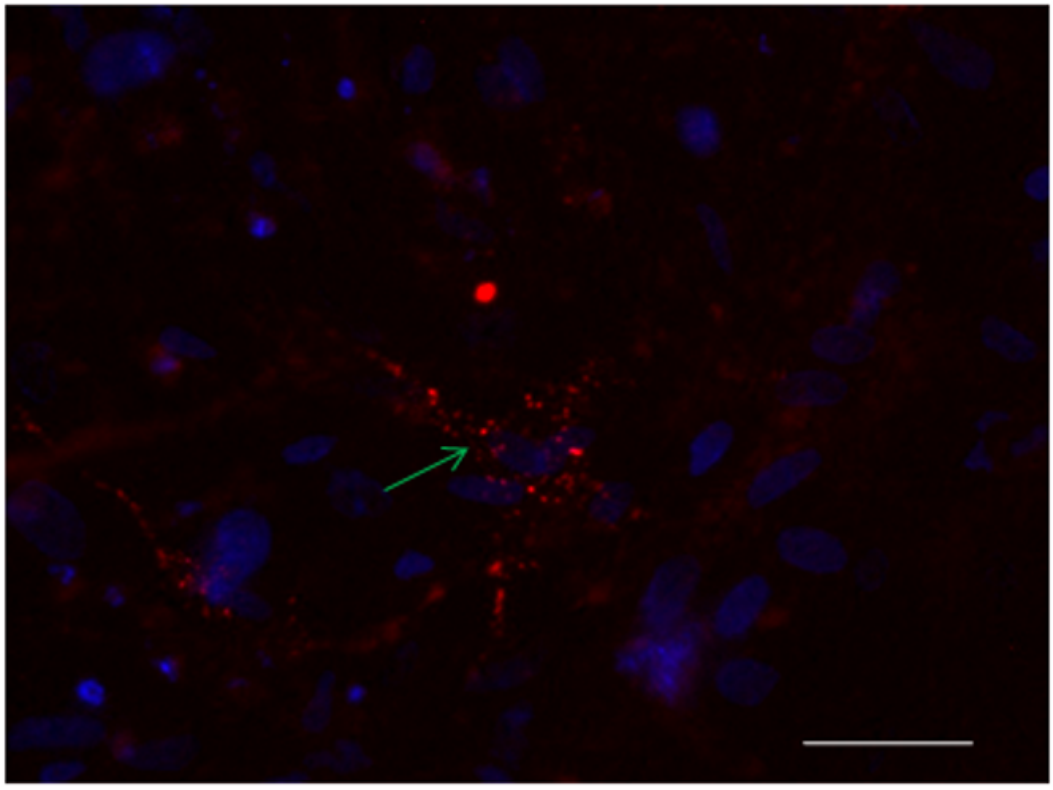


**Figure 1. Successful dosing of extracellular vesicles on cells.** Maleimide-stained EVs from LOC effluent were visualized after 72 h perfusion period using fluorescence microscopy. Representative images of the fluorescently labelled EVs (red).

3 **Effluent collection (also applicable for other organ-on-chip derived effluent):**

1. Prepare Eppendorf tubes to keep record of samples.
2. A single channel pipette can be used to collect outlet effluent from outlet reservoir and inlet media from inlet reservoir for each channel at a time from one pod (***see*, Note below).**

Note

Avoid direct contact with the reservoir “Vias” and change tips between sample collection to avoid cross-contamination.

3. Place the Eppendorf tubes with samples on ice immediately.

4. Alternatively, samples can be stored at -80°C for isolating EVs in future.

4 **Measurement of effluent volume:**

1. Weigh each tube individually. If samples were stored at -80°C, transfer the collected samples on ice and wait till they thaw.
2. Once thawed bring the samples to the weighing station while they are still on ice. Wipe away excess ice and water from the exterior of each tube prior to weighing. Record the weight of each tube in grams (g).
3. Subtract the average weight of an empty Eppendorf tube from the recorded values, to obtain the net weight of the collected samples (***see*, Note below**).

Note

Obtain average Eppendorf tube weight by averaging the weight of 3 empty Eppendorf tubes.

1. Using a 1 g = 1 mL correlation, convert the net weight of each tube to the volume of sample collected (i.e., 500 mg = 500 µL) for LOC media.
2. Take same volume of media before proceeding with EV isolation (***see*, Note below**).

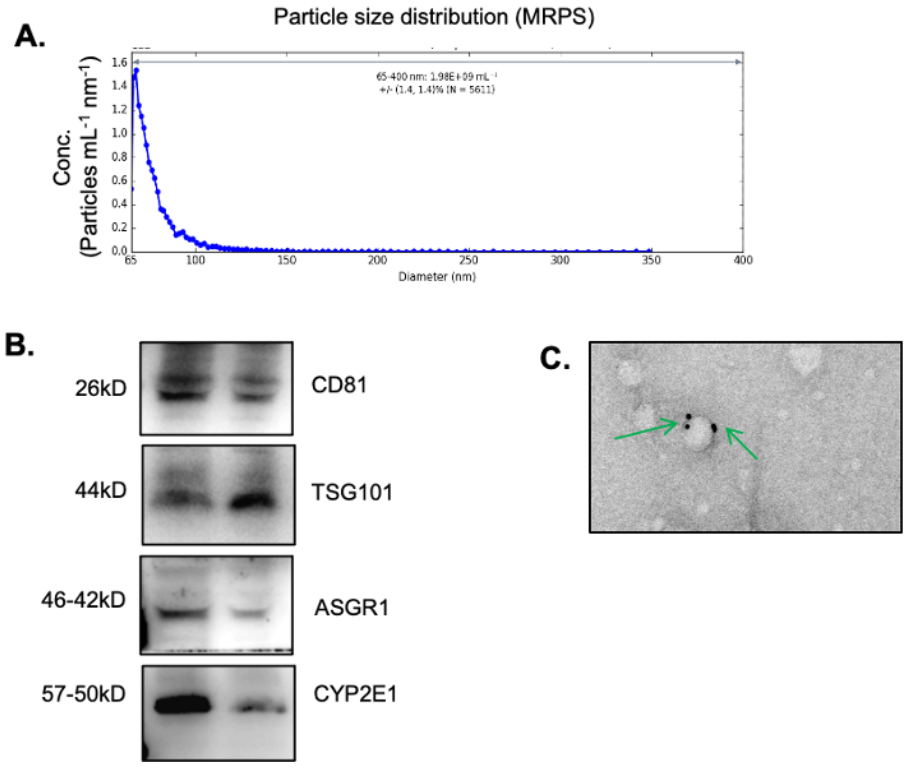
Note

Depending upon the experimental need, top channel and bottom channel effluent can either
analysed separately or combined.

3. For EV isolation and analysis, see chapter “Isolation and Characterization of Tissue and Cell-Derived Extracellular Vesicles and Non-Vesicular Extracellular Particles” for detailed method. EVs were characterized using microfluidic resistive pulse sensing (MRPS), Western Blotting, and transmission electron microscopy (TEM) (**Figure 2**).

Expected result

Figure 2.

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guidelines (**Fig. 2**). **(A)** For particle size distribution we performed Microfluidic Resistive Pulse Sensing (MRPS). **(B)** We performed immunoblot with canonical EV specific markers
(CD81 and TSG101) along with hepatocytes specific EV markers (CYP2E1 and ASGR1). **(C)** Finally, we visualized the typical cup shaped EV structure by transmission electron microscopy (TEM) (Green arrows indicate immuno-labelled CYP2E1 on EVs; scale bar used = 200 nm).

5 **Extracellular vesicle preparation for GC-MS-based metabolomics analysis:**

1. Collect 500 µL of effluent media from pod outlets into respective Eppendorf tubes.
2. Isolate EVs using automated SEC (see next chapter for detailed method).
3. Concentrate upto 100 µL.
4. Use 10 µL of EVs into an Eppendorf tube.
5. On ice, add 190 µL of ice cold Media Extraction Mix, to a final volume of 200 µL.
6. Vortex for 10 min at max speed at 4°C.
7. Centrifuge for 10 min at max speed at 4°C.

8. Take up to 200 µL of the media-extraction mix and place into respective fresh 1.5 mL Eppendorf tubes.
9. Dry on the speed vac for up to 2-3 hours.
10. Store at -80°C until ready to run on the GCMS.

## 6 Bulk RNA-Seq of kidney-chip cells treated with extracellular vesicles

1. Treat RPTECs and RMVECs in the kidney-chip with EVs as described.
2. After treatment with EVs for 72h, collect RPTECs and RMVECs in 600 µL lysis buffer from the RNA PureLink Mini Kit containing 1% 2-Mercaptoethanol.
3. Store lysed samples in lysis buffer at -80°C until RNA extraction.
4. For RNA extraction, thaw samples on ice.
5. Sonicate samples using an ultrasonic bath or 2 min (setting: 30 s: on, 30 s: off).
6. Extract RNA using the PureLink RNA Mini Kit following Manufacturer's instructions except for the following modifications:

- When loading sample onto the spin column, spin sample at 2,000 *x g* for 1 min, followed by 12,000 *x g* for 15s using a benchtop centrifuge at room temperature.
- When eluting RNA sample, conduct two serial elutions: Add 10 µL molecular grade water, incubate on the spin column for 10 minutes at room temperature, and spin sample at 2000 *x g* for 1 min, followed by 12,000 *x g* for 15 s using a benchtop centrifuge at room temperature. Repeat once with an additional 10 µL of molecular grade water for a final elution volume of 20 µL.

7. Remove an aliquot of RNA for quality control using the Nanodrop and Bioanalyzer.
8. Store RNA samples at -80°C until library preparation for bulk RNA-Seq.
9. Assess RNA quantity and quality by measuring 260/280 and 260/230 absorbance ratios of RNA samples using the Nanodrop spectrophotometer.
10. Assess RNA quality and RNA integrity number (RIN) using the Agilent RNA 6000 Nano Kit and Bioanalyzer (**Figure 3A and B**).
11. Prior to library generation using ribodepletion, ensure that any contaminating DNA is removed from RNA samples by treatment with Ambion DNA-freeTM DNA Removal Kit. According to the manufacturer's instructions for routine DNase treatment.
12. Using up to 1000 ng treated RNA from the previous step as input, prepare libraries for bulk RNA-Seq using the KAPA RNA HyperPrep Kit with RiboErase (HMR) according to the manufacturer's instructions, and with the following protocol recommendations:

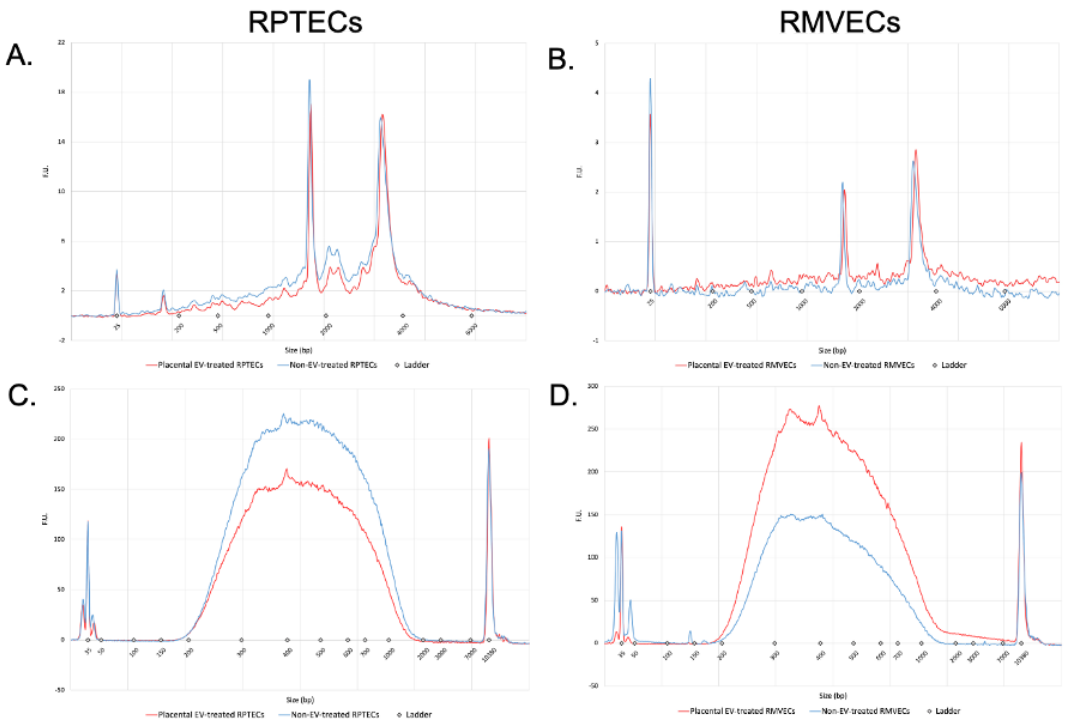
- Adjust RNA fragmentation time/temperature according to RNA quality:
  - RIN ≥ 9: 6 mins at 94 degrees
  - RIN 6-8.9: 6 mins at 85 degrees
  - RIN 4-5.9: 5 mins at 85 degrees
  - RIN 2-3.9: 4 mins at 85 degrees
  - RIN < 2: 3 mins at 85 degrees
- Use KAPA Unique Dual-Indexed Adapters for the adapter ligation step, adjusting adapter stock concentration based on both starting material quantity and

quality, per the manufacturer's recommendations.

- Adjust library amplification cycles based on quantity of starting material, per the manufacturer's recommendations, not to exceed 15 cycles.

13. Assess quality of RNA-Seq libraries using the Agilent High Sensitivity DNA Kit and Bioanalyzer (**Figure 3C and D**).
14. Multiplex samples for RNA-Seq using Illumina platform.

**Expected result**

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## Protocol references

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## Citations

**Step 2.2**

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