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Extracellular Vesicle Uptake Assay *via* Confocal Microscope Imaging Analysis

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Abstract

There is a need for practical assays to visualize and quantify the cells' extracellular vesicle (EV) uptake. EV uptake plays a role in intercellular communication in various research fields; cancer biology, neuroscience, and drug delivery. Many EV uptake assays have been reported in the literature; however, there is a lack of practical, detailed experimental methodology. EV uptake can be assessed by fluorescently labeling EVs to detect their location within cells. Distinguishing between internalized EVs in cells and the superficial EVs on cells is difficult, yet critical, to accurately determine the EV uptake. Therefore, an assay that efficiently quantifies EV uptake through three-dimensional (3D) fluorescence confocal microscopy is proposed in this work. Fluorescently labeled EVs were prepared using a nano-filtration-based microfluidic device, visualized by 3D confocal microscopy, and then analyzed through advanced image-processing software. The protocol provides a robust methodology for analyzing EVs on a cellular level and a practical approach for efficient analysis.

Introduction

Extracellular vesicles (EVs) are nano-sized, lipid membrane-bound particles that are categorized by their sizes: ectosomes (100-500 nm) and exosomes (50-150 nm)[1]. EVs contain various biomolecules, such as proteins, nucleic acids, and lipids. These biomolecules originate from the cells before being encapsulated as cargo and released into the extracellular space *via* EVs[1,2,3].

Due to the variety of their cargo, EVs are believed to play an active role in intercellular communication. The release and uptake of EVs by cells allow the transfer of biomolecules

between the cells[4,5]. The introduction of EV cargo to a cell may alter the recipient cell's functions and homeostatic state[4,5,6]. EVs are internalized through multiple pathways; however, the exact mechanisms have not been accurately demonstrated.

The majority of the EV uptake assays, such as genetic tagging, fluorescently label individual EVs[7]. The resulting signal can be measured by microplate photometer, flow cytometry, or microscopy, with each technology having substantial limitations. Microplate photometers, flow cytometry, or standard two-dimensional (2D) microscopy cannot distinguish between internalized and superficially attached EVs[8,9]. Additionally, the necessary sample preparation for each of these techniques may introduce additional issues to EV uptake evaluation. For example, lifting adhered cells with trypsin before EV uptake analysis may cleave some superficially attached EVs on the cell's surface[10,11]. Trypsin may also interact with the cell surface, affecting cell and EV phenotype. Additionally, trypsin may not detach superficial EVs entirely, skewing isolated populations.

To accurately label EVs with fluorescent dyes, additional wash steps are required to remove the residual dye[7]. Accepted isolation techniques can also contribute to false-positive signals due to coagulation that occurs during EV isolation. For example, serial ultracentrifugation (UC) is widely used to isolate EVs and remove the immobilized dye. However, UC may co-precipitate EVs, and the residual dye may lead to a false-positive signal[12,13]. Other nano-filtration methods, such as column-based filtration, are also widely used for non-immobilized dye removal. The complex nature of EVs and dye interacting within the column matrix may lead to incomplete removal of residual dye due to the molecular cut-off of the column being altered by the complex input[14,15,16].

The current protocol proposes a nano-filtration-based microfluidic device to isolate and wash fluorescently labeled isolated EVs. The nano-filtration-based microfluidic device can provide efficient filtration *via* fluid-assisted separation technology (FAST)[17,18]. FAST reduces the pressure drop across the filter, thus reducing potential aggregation between EVs and dyes. By efficiently removing residual dye, it is possible to enhance the quality of fluorescently labeled EVs and the assay's specificity.

Confocal microscopy can distinguish between internalized and superficially attached EVs on the cell surface and comprehensively investigate the cellular mechanisms of EV uptake in a spatiotemporal resolution[19,20,21,22,23,24,25]. For example, Sung et al. described the visualization of the exosome lifecycle using their developed live-cell reporter. The location of the internalized EVs was detected and analyzed using a confocal microscope in three-dimension (3D) and post-image processing tools[20]. Although the size of small EVs (40-200 nm) is below the resolution limit of the optical microscope, the fluorescently labeled EVs can be detected by confocal microscopy since the photodetector can detect the enhanced fluorescence emission. Therefore, the subcellular localization of the fluorescently labeled EVs within a cell can be precisely determined by acquiring multiple z-stacked images of the EVs and the surrounding cellular organelles.

Additionally, 3D reconstruction and post-data processing can provide further insight into the positioning of the internalized, superficial, and free-floating EVs. By utilizing these processes in conjunction with the time-lapse live-cell imaging offered by confocal microscopy, the level of EV uptake can be precisely evaluated, and the real-time tracking of EV uptake is also possible. Further, EV trafficking analysis can be performed using confocal microscopy by assessing the co-

localization of EVs with organelles, a first step to determine how internalized EVs are involved in the intracellular function. This protocol describes the methodology for performing an EV uptake assay using the nano-filtration-based microfluidic device[17,26], confocal microscopy, and post-image analysis.

Protocol

1. EV isolation and on-chip immuno-fluorescent EV labeling

1. Collection of cell culture media (CCM) and pre-processing of CCM for EV isolation
    1. Seed PC3 cells at 30% confluency in a 75 cm^2 cell culture flask. Allow control cells to grow to 90% confluency (~48 h) in standard media and cell-line-specific supplements.
    NOTE: To prevent EV-containing components from affecting cellular uptake (i.e., fetal bovine serum), use exosome-depleted media and supplements.
    2. Harvest the CCM.
    3. Centrifuge the CCM at 1000 x *g* for 10 min at room temperature (RT) to pellet any unattached cells and large debris harvested with the media. Transfer the supernatant to a new conical tube.
    4. In the new tube, centrifuge the supernatant at 10,000 x *g* for 20 min at 4 °C to pellet smaller debris and apoptotic bodies remaining in the media. Some larger EVs will pellet. Transfer the supernatant to a new tube.
    5. Filter the supernatant through a 0.45 µm hydrophilic Polyvinylidene fluoride (PVDF) membrane syringe filter.
    NOTE: If not immediately processing CCM for EV isolation, store the pre-processed CCM at -80 °C until isolation is performed. If frozen, limit freeze-thaw cycles to one.
2. EV isolation from CCM using a nano-filtration based microfluidic device
    1. If frozen, completely thaw CCM and vortex for 30 s before step 1.2.2.
    2. Inject 1 mL of pre-processed CCM (step 1.1) into the sample chamber of the nano-filtration-based microfluidic device (see **Table of Materials**)[17,26].
    NOTE: Follow the standard operating procedure for the nano-filtration-based microfluidic device[17,26].
    3. Spin at 3000 rpm for 10 min in the bench-top spinning machine (see **Table of Materials**) to operate the microfluidic device.
    NOTE: If CCM remains on the sample chamber following the initial run, perform additional spins until all CCM has emptied from the sample chamber.
    4. Remove the fluid from the waste chamber by pipetting and repeat steps 1.2.1, 1.2.2, and 1.2.3 twice.
    NOTE: In total, 3 mL of CCM will be processed for EV isolation.
    5. Inject 1 mL phosphate-buffered saline (PBS) into the sample chamber to wash the isolated EVs. Spin in the bench-top spinning machine for operating the microfluidic device as mentioned in step 1.2.3. Locate the pure EVs on the membrane of the device.
    NOTE: The quality of EVs isolated from the nano-filtration based microfluidic device, specifically, was confirmed and compared to the conventional UC method by transmission electron microscopy (TEM),

scanning electron microscope (SEM), nanoparticle tracking analysis (NTA), structured illumination microscopy, enzyme-linked immunosorbent assay and real-time PCR in the previous research[17,26].

3. Immunofluorescent labeling of EV using nano-filtration based microfluidic device (**Figure 1**)

    1. Select an EV-specific antibody according to the purpose of the assay (see **Table of Materials**).
    NOTE: Certain antibodies may interfere with ligand binding sites specific to EV-uptake pathways (i.e., endocytosis).

    2. Inject 1 µg/mL of the EV-specific antibody into the elution hole of the device containing 100 µL of isolated EVs.

    3. Incubate for 1 h in the dark at RT on a plate shaker to ensure the even distribution of the antibody across the sample.

    4. Attach an adhesive tape to the elution hole. Inject 1 mL PBS into the sample chamber to wash out any residual antibodies.

    5. Spin the device at 3000 rpm until the sample chamber is empty. Remove any fluid from the waste chamber by pipetting. Inject 1 mL PBS into the sample chamber.
    NOTE: Fluorescently labeled EVs will be located in the membrane chamber.

    6. Pipette the fluorescently labeled EVs (**Figure 2**) from the membrane chamber to an amber tube. Block from light until use.

2. Incubation of the cells with fluorescently labeled EVs for the EV-uptake assay

1. Target cell seeding and culture on the cell-culture compatible dishes.

    1. Seed 1 x 10^4 PC3 cells into the microslide 8-well plate (9.4 x 10.7 mm for each well) with 0.2 mL of media or 4 x 10^4 PC3 cells into a 35 mm dish with 1 mL of media. Plate the cells into a cell-culture compatible dish consisting of a thin coverslip (thickness: 0.18 mm).
    NOTE: The thin coverslip minimizes the adverse scattering of light.

    2. Allow cells to adhere overnight in optimal cell-culture conditions (37 °C, 5% CO_2 concentration, 90% humidity).

    3. Wash adhered cells twice with exosome-depleted media (described in step 1.1.1).

2. Cell incubation with fluorescently labeled EVs.

    1. Measure the concentration of fluorescently labeled EVs (step 1.3.5) by nanoparticle tracking analysis (NTA, **Supplementary Figure 1**). Determine the optimal concentration of fluorescently labeled EVs to be added to the cultured cells (step 2.1.2.).

    2. Dilute the fluorescently labeled EVs with exosome-depleted media to match the desired concentration measured in step 2.2.1. (i.e., 7.80 x 10^9 EVs (in NTA value) in 200 µL of exosome-depleted media.)

    3. Add the diluted EVs (Step 2.2.2) to the adhered target cells prepared at 2.1.2. Incubate for experimental time (i.e., 4, 8, or 12 h).

4. Wash cells thrice with exosome-free media to remove any non-internalized EVs.
   NOTE: Optional: Cells can be fixated following wash.
5. Label the cytoplasm of the adhered cells with 1 µg/mL of CMTMR ((5-(and-6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine) (see **Table of Materials**) and incubate in optimal cell-culture conditions (37 °C, 5% CO_2 concentration, 90% humidity).
   NOTE: Cell area dyes should fluoresce separately from labeled EVs to aid in determining the spatial location (internalized or superficial) of the spiked EVs during the EV uptake assay.
6. Wash labeled cells twice with exosome-depleted media to remove the residual dye. Add fresh exosome-depleted media to the cells in preparation for live-cell confocal imaging.

3. Confocal microscopy

1. To perform live-cell imaging, utilize an on-stage incubator to maintain optimal cell-culture conditions (37 °C, 5% CO_2 concentration, 90% humidity).
2. Place the prepared cells in the on-stage incubator.
3. Set the imaging parameters based on control samples.
   NOTE: Suggested control samples include: Fluorescently labeled EVs only, fluorescently labeled cells, unlabeled EVs, and unlabeled cells.
4. Determine the depth of the target cells and the range of stacking size in the z-direction to acquire 3D confocal images.
   NOTE: The thickness of a Z-stack is 1 µm. The confocal 3D image acquisition lasted 2 min 34 s (each Z-plane image acquisition took approximately 8 s; a total of twenty Z-stack images).
5. Set image acquisition to multiple z-stacked images of both cell-specific dye (i.e., red) and EV-specific dye (i.e., green) simultaneously (**Figure 3** and **Figure 4A**).

4. Image processing

1. Utilize automatic image-processing software to analyze the raw z-stacked confocal images and determine the EV uptake by cells (see **Table of Materials**).
2. Set thresholding parameters to the fluorescent signal of the cells and EV-specific dyes. Build the virtual surfaces of cells (**Figure 4A,B**).
   1. To build the virtual surfaces of cells, click the button **Add new Surfaces**.
   2. Select **Shortest Distance Calculation** as "Algorithm Settings" to use the provided algorithm by the software, then click **Next: Source Channel**.
   3. Select **Channel 2 - CMTMR** as "Source Channel" in this experiment.
   4. Select **Smooth** and put the appropriate value into "Surfaces Detail" for surface smoothing.
      NOTE: 0.57 µm in this experiment since 1 pixel represents 0.57 µm in raw imaging data.
   5. Select **Absolute Intensity** as "Thresholding."
   6. To automatically threshold the fluorescent image by the provided algorithm, click **Threshold (Absolute Intensity): The value is automatically set**.
   7. Select **Enable** as "Split touching Objects (Region Growing)" and put the value of estimated cell size into "Seed Points Diameter," 10.0 µm in this experiment. Then click **Next: Filter Seed Points**.

8. To configure the virtual cell surfaces, click **+ Add** button, then select **Quality** as "Filter Type." Threshold the appropriate value (210 in this experiment) for the low limit by a visual inspection and the maximum value (1485) for the upper limit, then click the **Finish** button.
   NOTE: A visual inspection means that a researcher can discriminate the cellular area from a raw fluorescent image.
9. Next, to build the virtual dots of EVs, click the button **Add new Spots**.
10. Select **Different Spot Sizes (Region Growing)** and **Shortest Distance Calculation** as "Algorithm Settings," then click **Next: Source Channel**.
11. Select **Channel 1 - Alexa Fluor 488** as "Source Channel" in this experiment.
12. Put the appropriate value into "Estimated XY Diameter" for the spot detection, **1 µm** in this experiment. Then, click **Next: Filter Spots**.
13. To configure the virtual EV dots, click **+ Add** button, select **Quality** as "Filter Type," and set "Lower Threshold" by a visual inspection, **100** in this experiment. Then, click the **Next: Spot Region Type** button.
14. Select **Absolute Intensity** as "Spot Regions Type," then click **Next: Spot Regions**.
15. To threshold, the region of EV dots, put the appropriate value into "Region Threshold" by a visual inspection, **100** as "Region Threshold" in this experiment.
    NOTE: A visual inspection means that a researcher can discriminate the EVs area from a raw fluorescent image.
16. Select **Region Volume** as "Diameter from," then click **Finish**.

3. Use the software's provided algorithms to split the grouped spots inside the built surface at step 4.2 (**Figure 4C, i-iv**).
   1. Click the built **Spots**, then go into **Filters**.
   2. Click **+ Add** button, then select **Shortest Distance to Surfaces Surfaces = Surface 1** as **Filter Type**, then click **Duplicate Selection to new Spots** button. The lowest threshold (-7.0 in this experiment) for the low limit and the appropriate value (-0.5) for the upper limit.
      NOTE: Set the upper limit with the estimated radius of **Spots**. In this experiment, the estimated diameter of **Spots**,i.e., EV dots, was set to **1 µm** in step 4.2.11.; thus, the upper limit can be **0.5**.
4. Automatic count of EVs inside the cells
   NOTE: The software will automatically count the number of EVs inside the cells, indicating the number internalized by the target cells.
   1. Click the built **Spots 1 selection [Shortest Distance to Surfaces Surfaces = Surfaces 1 between -7.00 and -0.500].**
   2. Go to the **Statistics**, and export the value from "Total Number of Spots"
      NOTE: The software's provided algorithms will automatically calculate the number and volume of cells.

5. Determine the yield of EV uptake per incubation period based on the above-calculated values (**Figure 5**).
    1. To obtain the number of cells, click the built **Surfaces 1**, then go to the **Statistics**, and export the value of "Total Number of Surfaces" from **Overall**.
    2. Go to the **Detailed** into **Statistics** to export the **Volume** from **Detailed**.

Representative Results

Using a nano-filtration-based microfluidic device, EVs were isolated from PC3 CCM and labeled with a fluorophore-conjugated EV-specific (CD63) antibody (**Figure 1**). The labeled EVs were successfully visualized by the 3D confocal microscopy (**Figure 2**). The labeled EVs were incubated with cells for several hours in exosome-depleted media. Following incubation, cells were washed with exosome depleted media. The remaining EVs were internalized or adhered to cells during incubation. The cell area was labeled. Internalized EVs were visualized as puncta[19,20,24,27] and an individual EV (**Figure 3** and **Figure 4A**). Post-processing of these images allows the visualization and quantification of EV internalization into the cells (**Figure 4**). The steps in conjunction allow accurate EV uptake assay performed efficiently. **Figure 5** shows the representative results of the EV uptake assay. The assay indicates that the level of EV uptake is dependent on the length of the incubation period. The procedure allows for the systematic exclusion of non-internalized EVs (**Figure 4C**) to precisely mea+sure the number of internalized EVs. The size distribution of internalized EV dots was calculated (**Figure 6**). Furthermore, the number of internalized EVs can be normalized to the recipient cells' volume to determine the actual rate of EV uptake for the specific cell. Normalization accounts for the heterogeneous cell size and represents the number of internalized EVs regarding the cellular surface area. Cellular surface area is defined as the cell area in contact with EV-spiked, exosome-depleted culture media during incubation.

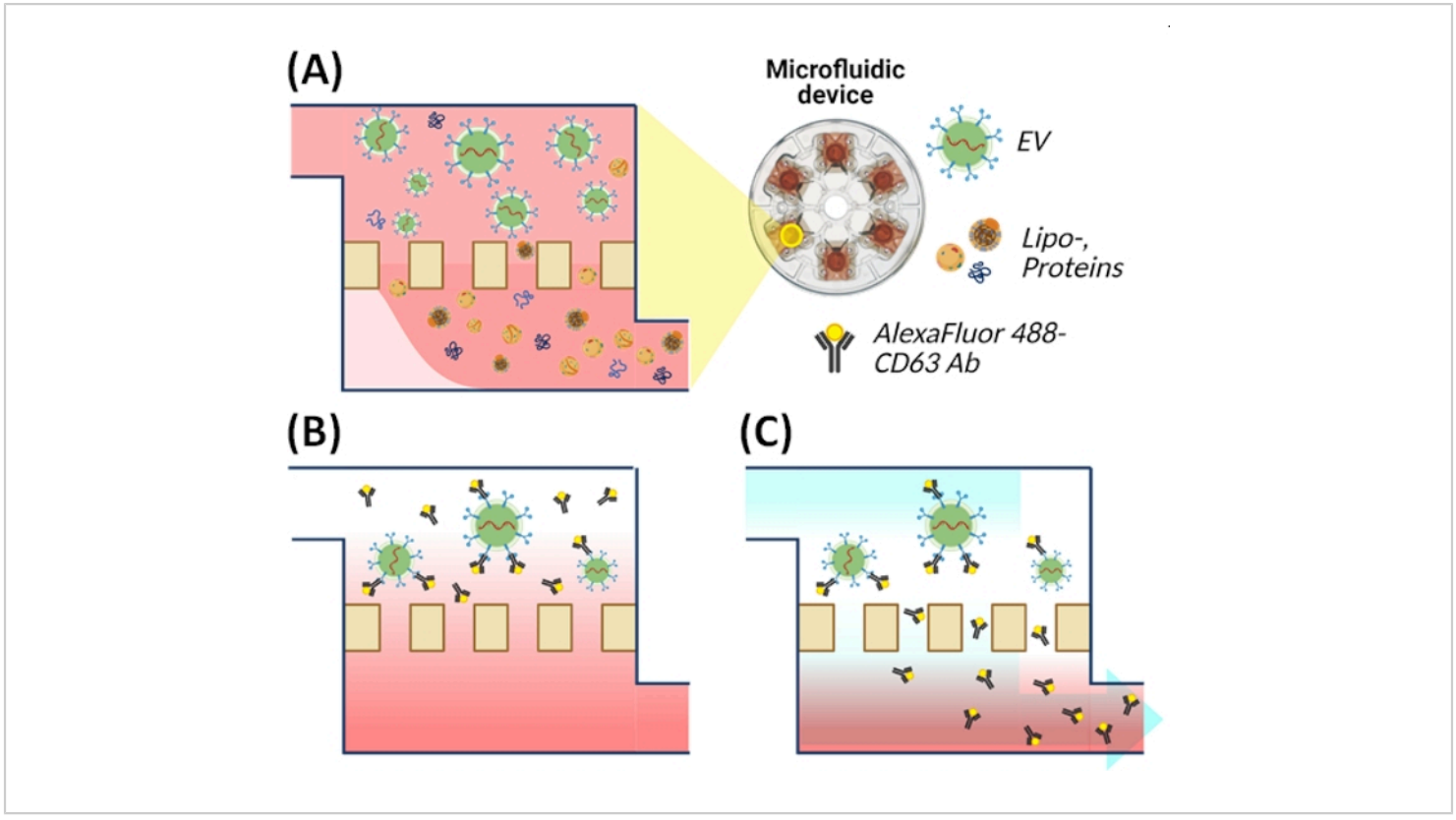

Figure 1: Schematic illustration of the EV isolation and on-chip labeling using a nano-filtration-based microfluidic device. (**A**) EVs isolation from CCM. (**B**) On-chip Immunofluorescent labeling of EVs. (**C**) Removal of unbound antibodies.

Please click here to view a larger version of this figure.

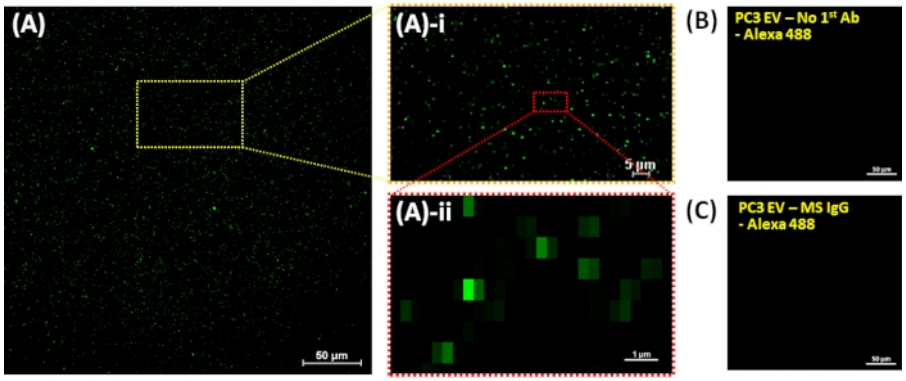

Figure 2: Imaging of fluorescently labeled EVs. The fluorescently labeled (anti-CD63-Alexa Fluor 488) EVs were detected using the confocal microscope (40x objective). (**A**) Positive sample (anti-CD63-Alexa Fluor 488 labeled EVs). (**B**) Negative control 1 for the EV labeling (EVs with 2nd antibody (Alexa Fluor 488) only, without 1st antibody). (**C**) Negative control 2 (EVs with the mouse (MS) IgG antibody and 2nd antibody (Alexa Fluor 488)). Please click here to view a larger version of this figure.

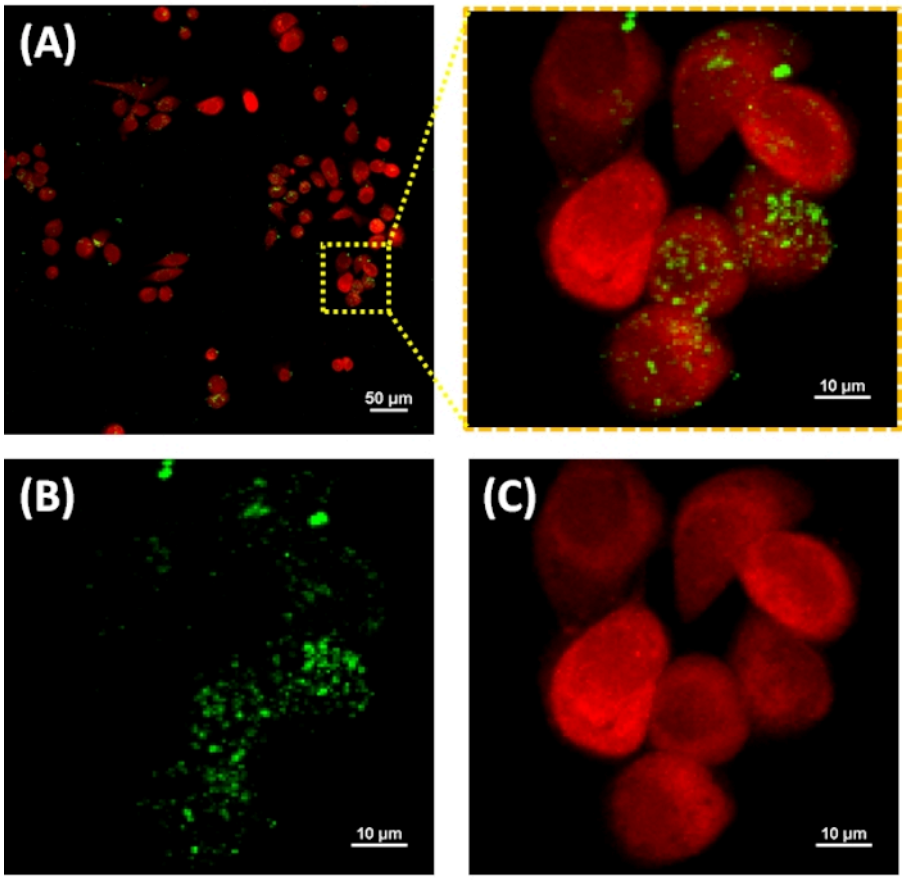

Figure 3: Imaging of the internalized EVs into cells in a 2D image. (**A**) The fluorescently labeled (anti-CD63-Alexa Fluor 488, green) EVs and the cells (CMTMR, red) were detected by using the confocal microscope (20x objective) after the incubation. (**B**) A separate image of the fluorescently labeled EVs only. (**C**) A separate image of the fluorescently labeled cells only. The excitation/emission laser wavelengths for CMTMR and Alexa Fluor 488 are 560.6/595 (±50) nm and 487.8/525 (±50) nm. Laser power settings are 3.0 % for CMTMR and 10.0 % for Alexa Fluor 488. Please click here to view a larger version of this figure.

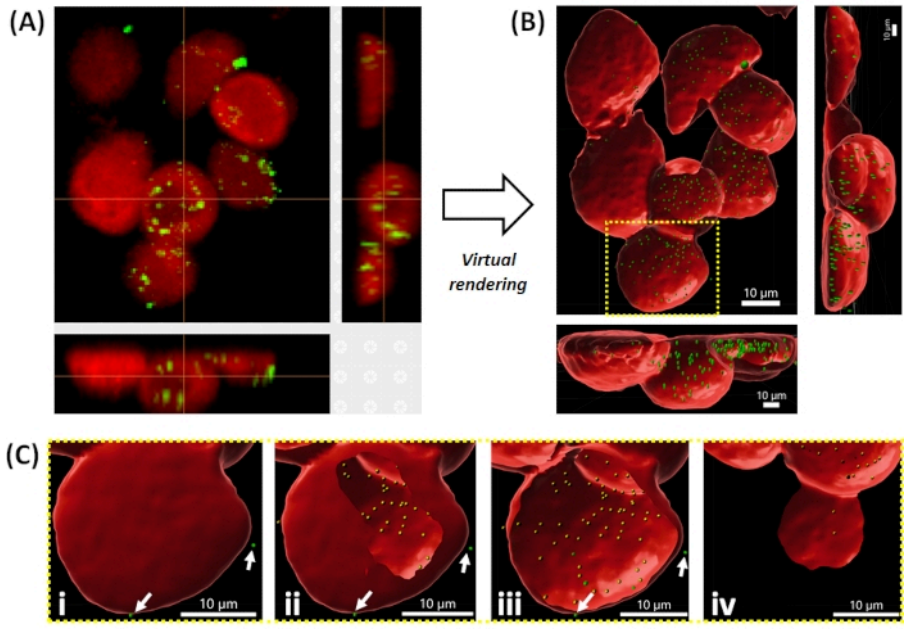

Figure 4: Quantification of the internalized EVs by the post-imaging process. (**A**) Raw confocal image obtained from the EV-uptake assay. (**B**) Virtual rendering of the EVs as a dot (green) and the cells as a surface (red) by using the image-processing software. (**C, i-iv**) Discrimination of the internalized EVs (yellow dots) and non-internalized EVs (green dots, white arrow) using the software provided algorithm. Please click here to view a larger version of this figure.

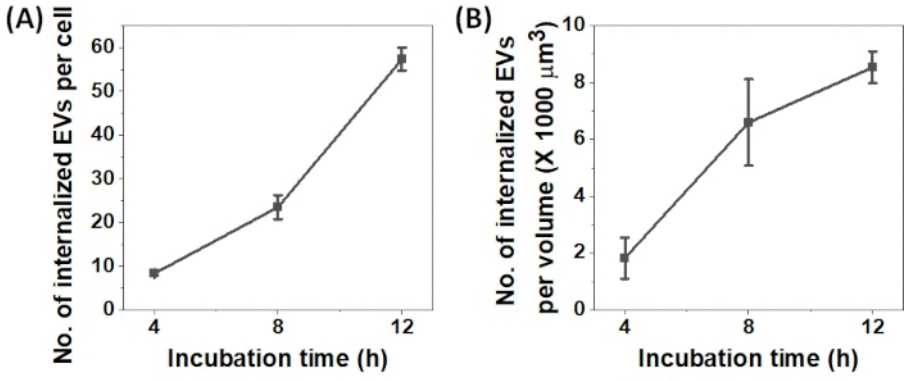

Figure 5: The amount of EV uptake as a function of incubation time. (**A**) The number of internalized EVs per cell. (**B**) The number of internalized EVs per cell volume. The number of internalized EVs was increased depending on the incubation time. Please click here to view a larger version of this figure.

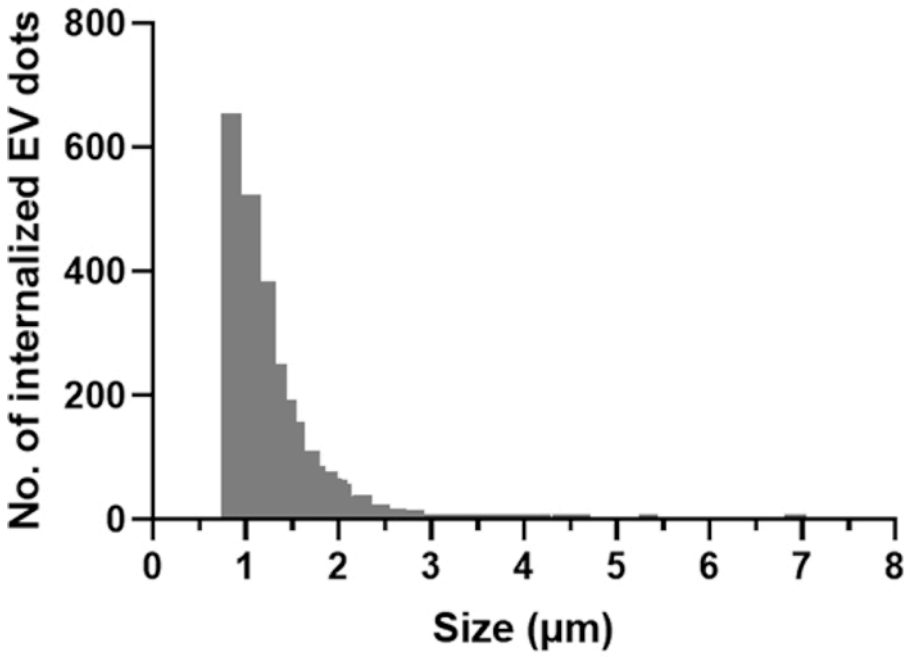

Figure 6: The size distribution of internalized EV dots. The size of EV dots was measured and plotted to a distribution. Please click here to view a larger version of this figure.

Supplementary Figure 1: NTA measurement of anti-CD63-Alex Fluor 488 labeled EVs. Please click here to download this File.

Supplementary Figure 2: The amount of co-localized EVs with lysosomes as a function of incubation time. Please click here to download this File.

Supplementary Figure 3: The amount of EV uptake as a function of incubation time. (**A**) The number of internalized EVs per cell. (**B**) The number of internalized EVs per cell volume. The number of internalized EVs was increased depending on the incubation time. The EV sample was labeled by RNA staining dye (see **Table of Materials**). Please click here to download this File.

Discussion

An EV uptake assay based on 3D fluorescence imaging *via* confocal microscopy provides an efficient methodology and sensitive analysis. This fluorescent EV labeling facilitates the visualization of EVs and successfully performs a precise EV uptake assay. Previous methods for labeling EVs and removing the residual dye have been reported by removing precipitation using ultracentrifugation (UC); however, UC may co-precipitate EVs, and the immobilized dye may lead to a false-positive signal[12,13]. Nano-filtration-based microfluidic devices eliminate this co-precipitation of EVs and dye, thus enhancing the quality of the fluorescently labeled EVs and the assay's specificity. EV uptake was measured by volumetric analysis to distinguish and quantify the internalized EVs separate from superficial EVs on the cell surface. The

volumetric analysis of EV uptake allows for the normalization of EV uptake by cell size. The live-cell EV uptake assay was achieved by utilizing the on-stage confocal microscope incubator. The protocol applies to studies requiring live cell cultures and EVs. Real-time intracellular EV tracking can be performed across a 3D window. Additionally, EV trafficking analysis of spatial-temporal resolution along with the co-localization of EVs with subcellular organelles can be achieved through the protocol (**Supplementary Figure 2**). The protocol described here is a powerful tool for the cellular analysis of EVs.

Despite all advantages of the protocol, there are potential limitations. The nano-filtration microfluidic device utilized in this study may co-isolate other contaminants during EV isolation. Low-density lipoproteins are similar in size to EVs and may be caught in the membrane during isolation[28]. Although EV-specific markers can specify EVs, the co-isolated contaminants may affect downstream analysis. In this manuscript, EVs were labeled with a CD63 conjugated Alexa Fluor 488 dye. This labeling increases the specificity for EV detection; however, it also binds the EV surface molecule and increases competitive binding. Additionally, the level of EV uptake may be dependent on the cell line and CCM. The labeling methods detailed in this protocol can be adapted to other staining dyes such as lipophilic dyes, cytosolic dyes, and RNA dyes (**Supplementary Figure 3**). The protocol uses a light scanning confocal microscope (LSCM) to detect the tiny signal of EVs. LSCM relies on intense lasers and, as a result, living cells may be damaged or altered by long-term laser excitation. The fast acquisition speed can decrease photodamage by utilizing a spinning disc confocal microscope (SDCM). SDCM can also be used in EV tracking that requires short time-lapse imaging to visualize short-distance movements.

Despite these potential limitations, the proposed protocol provides an efficient method for EV-uptake assessment and has the potential for further live-cell EV-tracking analysis.

Disclosures

Y.-K. Cho is an inventor of the patents on the nano-filtration-based microfluidic device, Exodisc, which are licensed to Labspinner (Ulsan, Korea). All other authors have nothing to disclose.

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