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TITLE:

Extracellular Vesicle Uptake Assay *via* Confocal Microscope Imaging Analysis

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SUMMARY:

Extracellular vesicles (EVs) contribute to cellular biology and intercellular communications. There is a need for practical assays to visualize and quantify EVs uptake by the cells. The current protocol proposes the EV uptake assay by utilizing three-dimensional fluorescence imaging *via* confocal microscopy, following EV isolation by a nano-filtration-based microfluidic device.

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[1-3].

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[4-6]. 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[14-16].

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[19-25]. 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

1.1. Collection of cell culture media (CCM) and pre-processing of CCM for EV isolation

1.1.1. 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.

1.1.2. Harvest the CCM.

1.1.3. 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.

1.1.4. 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.

1.1.5. 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.

1.2. EV isolation from CCM using a nano-filtration based microfluidic device

1.2.1. If frozen, completely thaw CCM and vortex for 30 s before step 1.2.2.

1.2.2. 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].

1.2.3. 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.

1.2.4. 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.

1.2.5. 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].

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

1.3.1. 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).

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

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

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

1.3.5. 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.

1.3.6. 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

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

2.1.1. 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.

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

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

2.2. Cell incubation with fluorescently labeled EVs.

2.2.1. 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.).

2.2.2. 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.)

2.2.3. 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).

2.2.4. Wash cells thrice with exosome-free media to remove any non-internalized EVs.

NOTE: Optional: Cells can be fixated following wash.

2.2.5. 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.

2.2.6. 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

3.1. To perform live-cell imaging, utilize an on-stage incubator to maintain optimal cell-culture conditions (37 °C, 5% CO_2 concentration, 90% humidity).

3.2. Place the prepared cells in the on-stage incubator.

3.3. 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.

3.4. 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).

3.5. 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

4.1. Utilize automatic image-processing software to analyze the raw z-stacked confocal images and determine the EV uptake by cells (see **Table of Materials**).

4.2. Set thresholding parameters to the fluorescent signal of the cells and EV-specific dyes. Build the virtual surfaces of cells **(Figure 4A,B)**.

4.2.1. To build the virtual surfaces of cells, click the button **Add new Surfaces**.

4.2.2. Select **Shortest Distance Calculation** as "Algorithm Settings" to use the provided algorithm by the software, then click **Next: Source Channel**.

4.2.3. Select **Channel 2 – CMTMR** as "Source Channel" in this experiment.

4.2.4. 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.

4.2.5. Select **Absolute Intensity** as "Thresholding."

4.2.6. To automatically threshold the fluorescent image by the provided algorithm, click **Threshold (Absolute Intensity): The value is automatically set**.

4.2.7. 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**.

4.2.8. 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.

4.2.9. Next, to build the virtual dots of EVs, click the button **Add new Spots**.

4.2.10. Select **Different Spot Sizes (Region Growing)** and **Shortest Distance Calculation** as "Algorithm Settings," then click **Next: Source Channel**.

4.2.11. Select **Channel 1 – Alexa Fluor 488** as "Source Channel" in this experiment.

4.2.12. Put the appropriate value into "Estimated XY Diameter" for the spot detection, **1 µm** in this experiment. Then, click **Next: Filter Spots**.

4.2.13. 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.

4.2.14. Select **Absolute Intensity** as "Spot Regions Type," then click **Next: Spot Regions**.

4.2.15. 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.

4.2.16. Select **Region Volume** as "Diameter from," then click **Finish**.

4.3. Use the software's provided algorithms to split the grouped spots inside the built surface at step 4.2 **(Figure 4C, i-iv)**.

4.3.1. Click the built **Spots**, then go into **Filters**.

4.3.2. 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**.

4.4. 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.

4.4.1. Click the built **Spots 1 selection [Shortest Distance to Surfaces Surfaces = Surfaces 1 between -7.00 and -0.500].**

4.4.2. 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.

4.5. Determine the yield of EV uptake per incubation period based on the above-calculated values (**Figure 5**).

4.5.1. 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**.

4.5.2. 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 LEGENDS:

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.

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)).

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.

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.

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.

Figure 6: The size distribution of internalized EV dots. The size of EV dots was measured and plotted to a distribution.

Supplementary Figure 1: NTA measurement of anti-CD63-Alex Fluor 488 labeled EVs.

Supplementary Figure 2: The amount of co-localized EVs with lysosomes as a function of incubation time.

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**).

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.

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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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25. Durak-Kozica, M., Baster, Z., Kubat, K., Stępień, E. 3D visualization of extracellular vesicle uptake by endothelial cells. *Cellular & Molecular Biology Letters.* **23**, 57 (2018).
26. Sunkara, V. et al. Fully Automated, Label-Free Isolation of Extracellular Vesicles from Whole Blood for Cancer Diagnosis and Monitoring. *Theranostics.* **9** (7), 1851-1863 (2019).
27. Li, H. et al. Internalization of trophoblastic small extracellular vesicles and detection of their miRNA cargo in P-bodies. *Journal of Extracellular Vesicles.* **9** (1), 1812261 (2020).
28. Dong, L. et al. Comprehensive evaluation of methods for small extracellular vesicles separation from human plasma, urine and cell culture medium. *Journal of Extracellular Vesicles.* **10**, e12044 (2020).

Figure 1

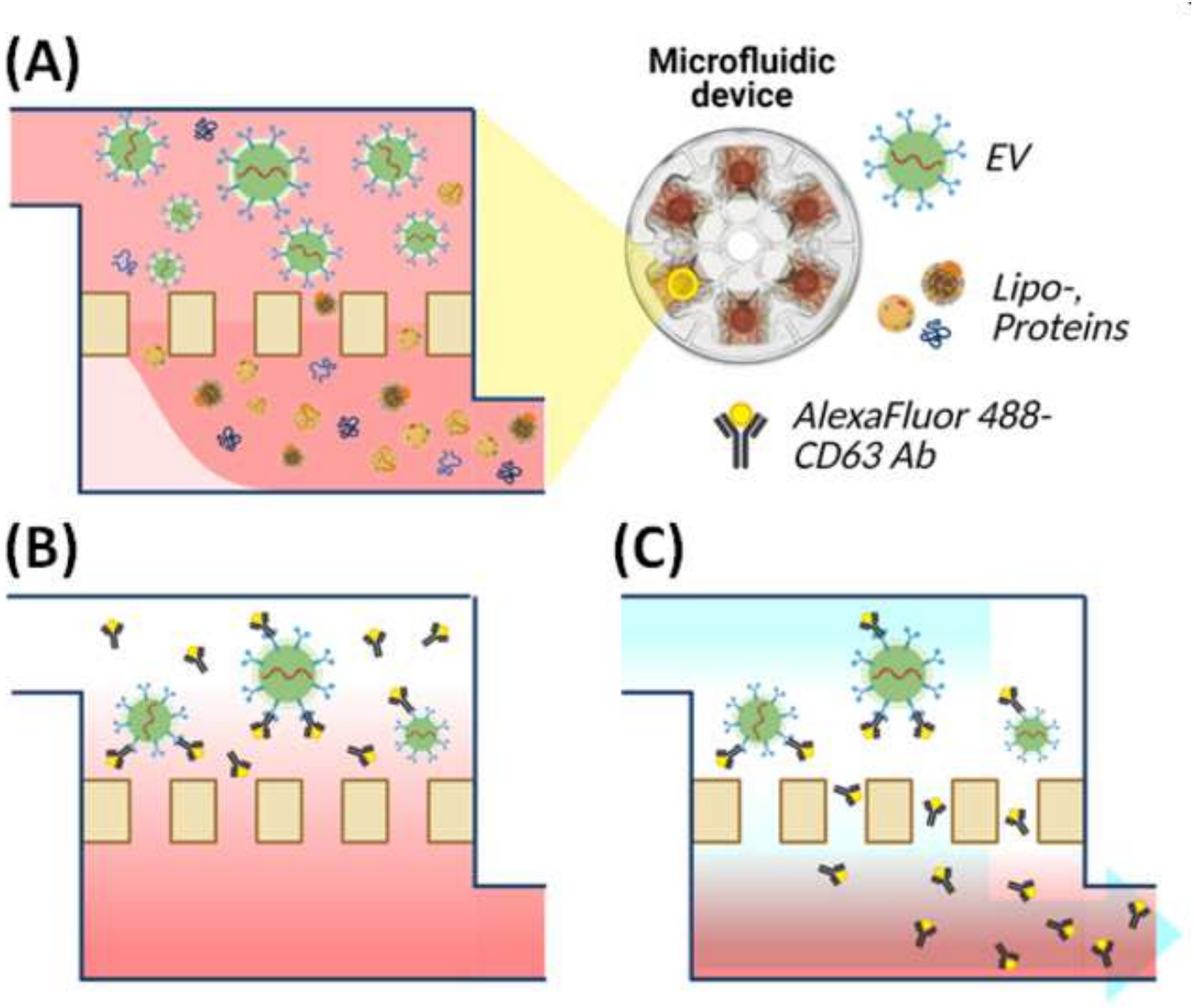


Figure 2

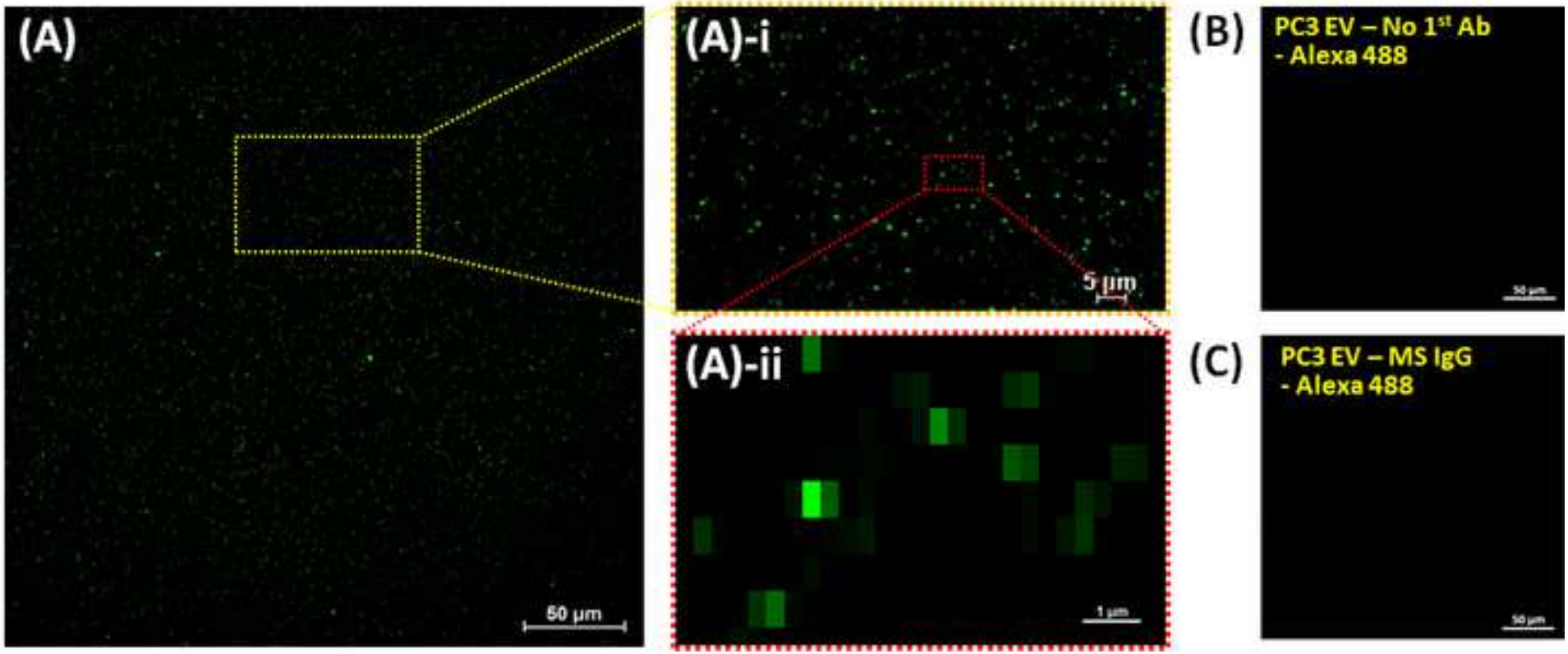


Figure 3

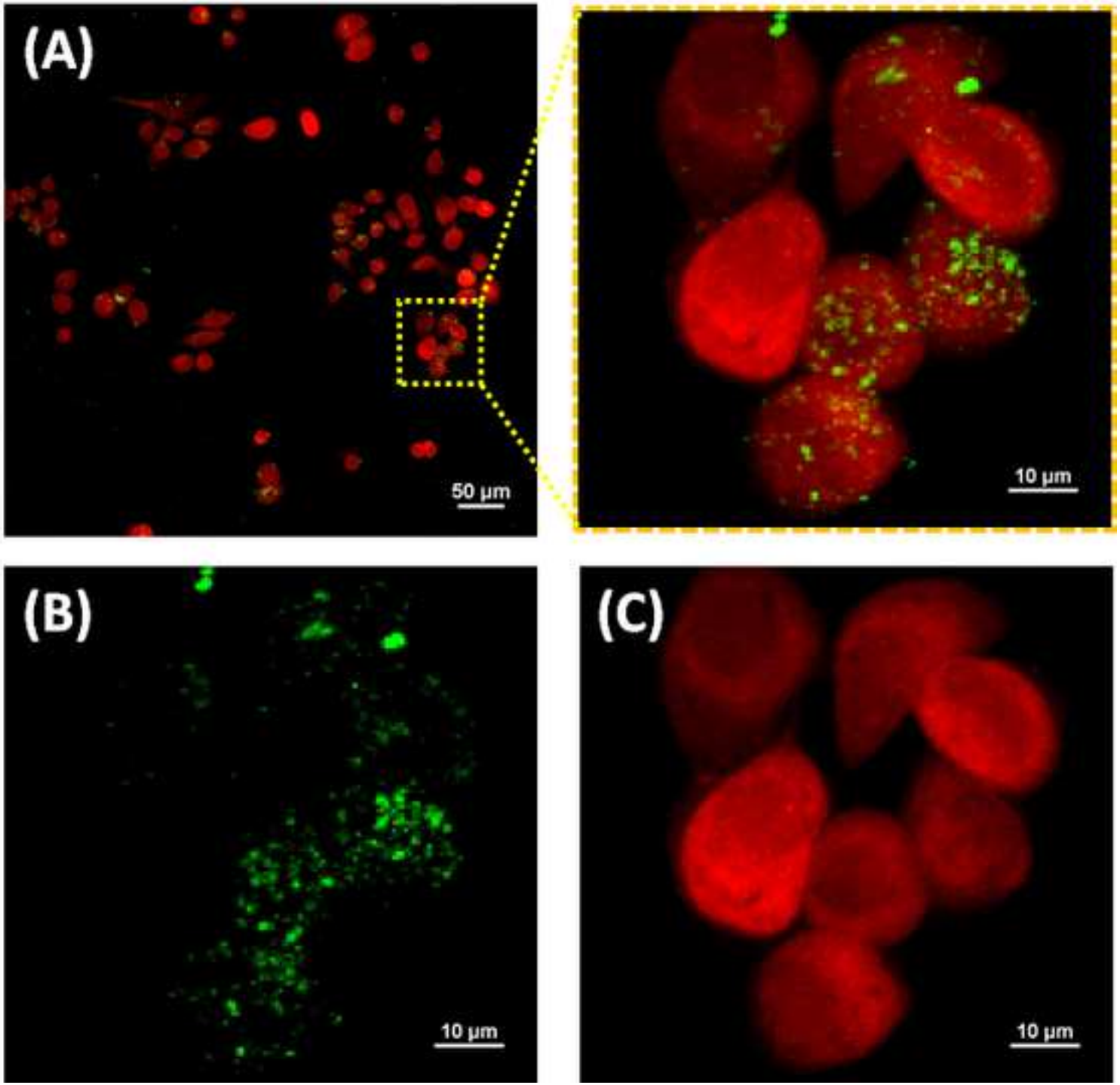


Figure 4

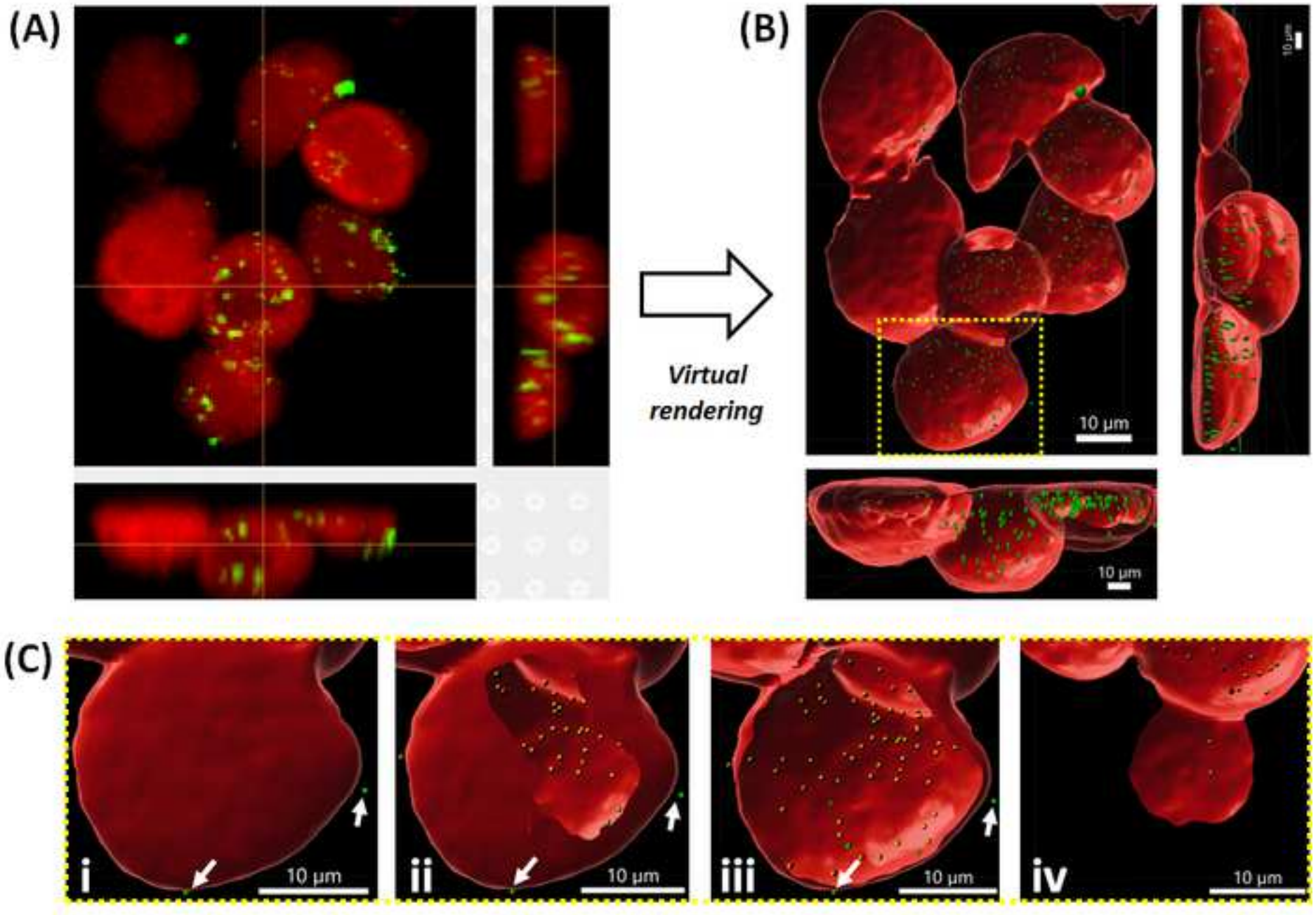


Figure 5

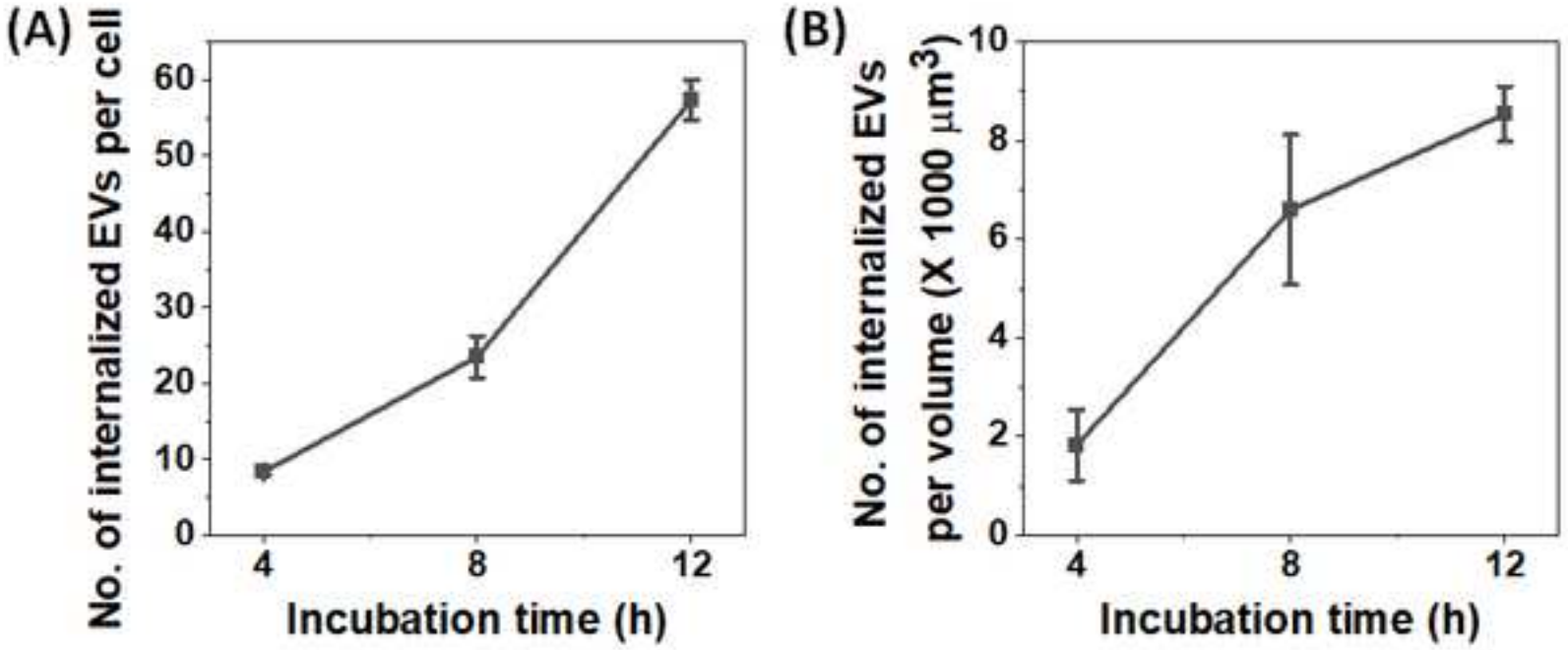
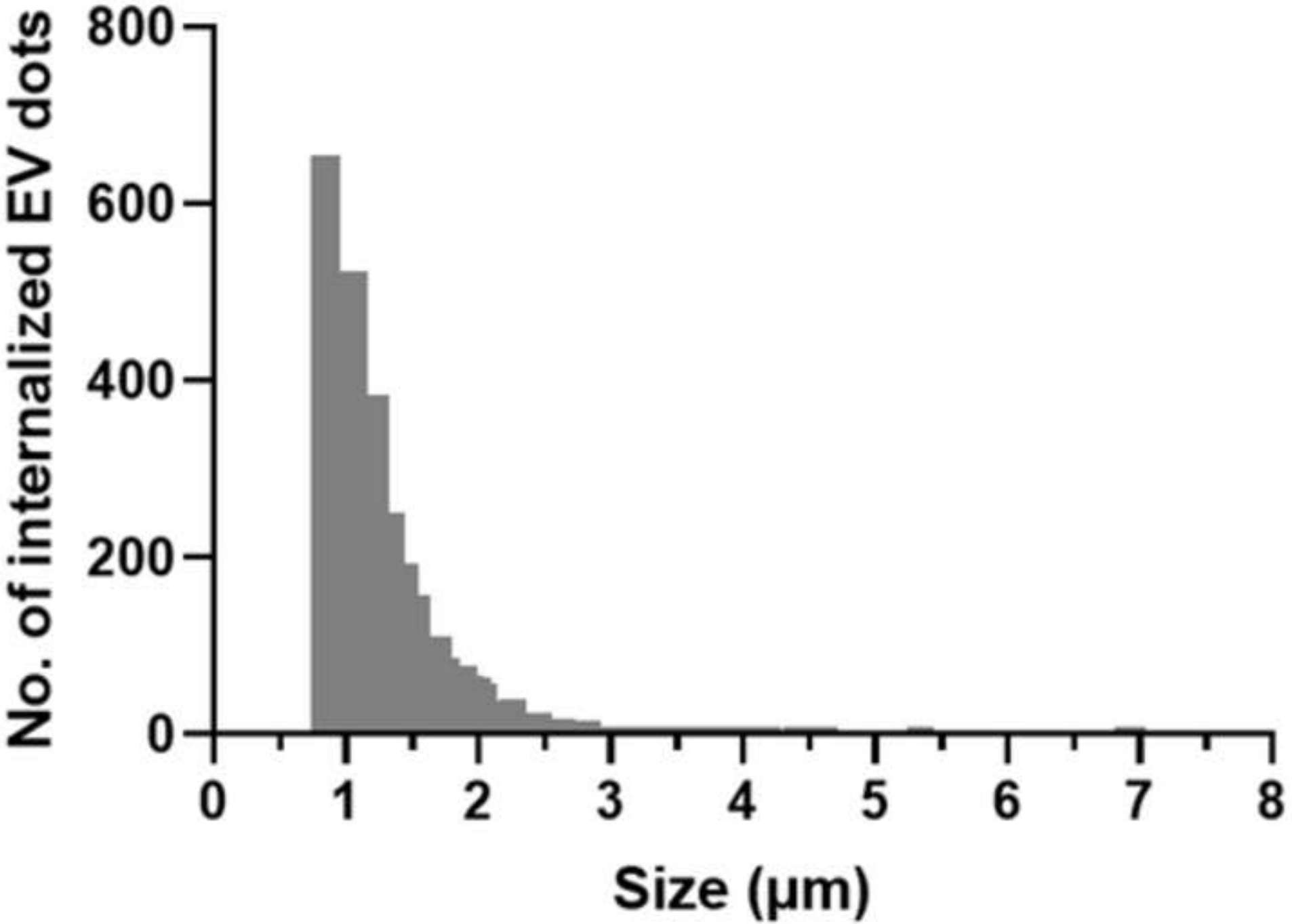


Figure 6

Click here to access/download;Figure;R2_Figure 6.psd



Editorial comments:

Changes to be made by the Author(s):

1. Please thoroughly proofread the manuscript.
2. Please submit each figure individually as a vector image file to ensure high resolution throughout production.
3. Also please designate the sub-figures as (A), (B), etc. in capital letters and not in small letters.
4. In the manuscript text, it is mentioned Alexa Fluor-CD63 antibody, but in Figure 1, it is mentioned FITC-CD63 antibody. Please clarify.
5. Please include the description of Figure 5 and Figure 6 in the Results section.
6. In the x-axis description of the Figure 5, please write Incubation time (h) and not Incubation time (hours).
7. Please include the images provided as “Reviewer’s only” as supplementary figures and provide the description in the Results section and Figure legend accordingly. These are valid points to be included.

Response: We appreciate the valuable comments. We have revised the manuscript (marked as blue text) according to the editorial comments.

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns and all the editorial comments. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Dear Editor and Reviewers,

We want to thank you for your insightful comments in regards to our manuscript and granting us the extension to ensure we returned quality revisions. We believe that by correcting and addressing your concerns, that we've significantly improved our piece. We appreciate the time and effort you put into them and hope that you find our corrections to be adequate.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Thank you for bringing these errors and inconsistencies to our attention. We have re-reviewed our manuscript, correcting spelling and grammar. Additionally, we corrected abbreviations to be defined during their first appearance in the manuscript.

2. Please provide an email address for each author.

Apologies for this oversight. We have added email addresses for each author into the revised manuscript prior to the summary (Lines (L) 14 - L16)

3. Keep the word count of the summary under 50 words.

Our apologies for this oversight, we have edited the manuscript summary to be more concise with a word count below 50 words (L18 – L22).

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example

Thank you for this comment. We have removed all trademark symbols and company-specific names from our manuscript. We agree that commercially labeled devices show bias towards product and can take away from the proposed methodology. To correct this, we have described the Exodisc as a nano-filtration based microfluidic device and refer to reference 2. We have removed "Exodisc" from all parts of the manuscript.

5. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g.,

"we", "you", "our" etc.).

We have revised the manuscript to avoid the use of personal pronouns from the protocol and have limited use throughout the manuscript. Thank you for this suggestion.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We agree and have updated our protocol to be written in the imperative tense, eliminating any passive phrases (i.e. Should be). We have also decreased the number of “Note” additions included within the protocol section. Thank you for these instructions, we hope we have adequately revised the section to meet them.

7. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of "Exodisc" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.

We briefly touched on this in our response to Editor’s comment #4. We agree that the naming of Exodisc can be perceived as a bias or advertisement for the company. We hope that by replacing Exodisc with ‘nano-filtration based microfluidic device’ we will draw attention towards the proposed methodology for EV uptake analysis.

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Thank you for this suggestion. We agree that more detail is necessary in the protocol steps, specifically when describing the 3D image analysis software, in order for the manuscript to adequately accompany the video. We’ve added details, including software actions and settings, and believe that the protocol has significantly improved in reproducibility. We have included the details mentioned below:

9. 3.3: what are the parameters? How do you set them?
3.4: How do you determine the depth of the target cells and the range of stacking size? How do you acquire images?
4.2: How do you build the virtual surfaces of cells and EVs? What thresholding parameters do you

use?
Mention that you are using PC3 cells right in the beginning.

Thank you for these comments. We hope by adding the additional details, mentioned in response #8, that we were able to address each of these questions. We added additional details related to these points, as well as, others we felt required more clarity. Revisions can be seen in line #s; 3.3: L322 - L328, 3.4: L204 – L206, 4.2: L215 – L266). We also mentioned that we are using PC3 cells at the beginning (L159) to ensure readers were aware of cell type.

10. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.

Thank you for these corrections. We have re-formatted the manuscript according to these recommendations. Additionally, we have highlighted protocol text for inclusion in the scripted video.

11. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

Thank you for catching this error, we reviewed all images in the revised manuscript to ensure they include a scale bar with a defined figure legend.

12. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable).

We have reviewed all figures and tables to ensure they include measurement definitions, scale bras, and error bars in the revised manuscript. Thank you for this suggested improvement.

13. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Thank you for this comment. We have revised the discussion section to include these critical discussion points. Such revisions can be seen in line #s: L358 – L379.

_____

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In the present manuscript, Kim et al describe a method to fluorescently label extracellular vesicles (EVs) isolated from cell culture medium using a microfluidic device, and to quantify their uptake by cultured recipient cells by confocal microscopy.

Overall, the manuscript is well written and the method appears solid and clearly described, potentially representing a valuable tool for researchers in the EV field.

Major Concerns:

My major concern regards the specificity of the signal detected after EV labeling with the fluorescent antibody. Appropriate negative controls should be included to show that the signal observed is not due to unspecific labeling of non-EV particles or other contaminants in the sample.

This is an excellent point and we would like to thank the reviewer for the valuable comment. We have added the results of the negative control experiments into Figure 1. We hope that by including the results of a negative control that we are highlighting the specificity of the dye to EVs.

Although authors showed that the number of internalized EVs increases depending on the incubation time, it could theoretically be that, after internalization, EVs are retained into the endosomal compartment or directed into lysosomes for degradation or recycled to the cell surface, thus loosing part of the signals. Please, could the authors comment on this issue?

Thank you for the insightful comment and questions. We have also considered the degradation of internalized EVs by lysosome after EV-uptake and previously attempted to quantify the number of co-localized EVs in lysosomes. (Reviewer Only Figure 1). Our results suggest that internalized EVs were not fully degraded in the time periods ranging from 4 hours to 12 hours. We felt this preliminary data was beyond the scope of this EV-uptake specific manuscript but agree that the information is valuable when performing such assays. That you for the valuable comment.

Minor Concerns:

1. In point 1.1.1, the authors should clarify which is the number of starting EV-donor cells recommended to carry out the procedure and discuss if this method could be virtually applied to all types of cell cultures, i.e., rodent primary cultures and iPSCs, or it is exclusively recommended for cell lines.

Thank you for this suggestion. The number of starting EV-donor cells, PC3 cells, were determined by confluency at approximately 30% confluency in a single T75 flask. After 48 hours of PC3 cells cultivation, the number of PC3 cells reached approximately 90% confluency, and EVs were assessed. We added a few sentences to explain to clarify the starting quantities (L92-L95).

2. In 2.1.1, the sentence "Cells should match those used to harvest EVs from CCM" sounds inappropriate, since it might be very relevant to evaluate EV uptake by a target cell type which is different than the donor one but present in the same organ or tissue. Please, remove this sentence.

We appreciate the valuable comment and agree that the statement is not necessary to this general methodology. We removed the sentence in the revised manuscript.

3. In 2.2.2, please indicate the optimal final concentration of EVs to be obtained to perform the assay.

Thank you for catching this error, we added the final concentration of EVs which used to perform the assay (L174).

4. The strengths of this method are clearly highlighted in the manuscript. However, authors should also mention potential limitations and possible issues of the present protocol in the discussion section.

Thank you for drawing our attention to this oversight. We have added potential limitations and possible solutions into the discussion (L363-L376). By doing so, we feel the discussion is more comprehensive of the assay.

Reviewer #2:

Manuscript Summary:

This is a new manuscript by Kim and coworkers, to be published alongside a JoVE produced video. The manuscript describes a technology to extract, label, visualize and quantify extracellular vesicle (EV) uptake in live cells. Overall, they describe their own, commercially available microfluidics platform (YK Cho is part of the management team of LabSpinner, the company that makes the ExoDiscTM platform), and provide limited details on fluorescent imaging via confocal microscopy, and post-imaging processing. Without additional details and descriptions, this manuscript does not provide sufficient information for person with skills in the field, to replicate the experiments, or design their own.

Major Concerns:

Introduction:

1. The description of prior knowledge in examining vesicle uptake; a number of groups have used confocal microscopy and post-imaging processing to analyze EV uptake, labeling EVs with Exoglow and cells with either membrane dye or cytoplasmic dyes, or even dyes identifying organelles. What is added here?

We have added the discussion on the previous research about EV-uptake into the introduction (L56 - L74) and discussion section (L363 – L376).

Protocol

1. 1.1.1 I would add that if someone wanted to collect EVs from polarized monolayers, to grow them in trans-well plates and collect apical versus basal supernatant after the cells form a proper monolayer

Thank you for this suggestion. We agree that the proposed experiment (i.e., collecting EVs from polarized monolayers via trans-well plates to distinguish apical and basal supernatant) is an extension of this methodology and could be enhanced by this assay. We believe that such a specific experiment is too direct for such a manuscript, however, and prefer to describe a more common assay for these methods (i.e. EV collection from cells).

2. 1.2. Exodisc: I recommend providing references here to confirm that the technique was verified using EM, characterization of EVs by size, potential and cargo and compared to results by ultracentrifugation.

Thank you for this recommendation. We agree that providing references for Exodisc enhances the validity of the manuscript. In the revised manuscript, the two references are added and a few sentences are written to describe that the technique (i.e. Exodisc) was validated by the previous research (L130-L134)

3. 1.3.1 Please note that not all EVs from all cell lines/tissues are CD63 positive. If the Exodisc selects for EVs specifically and avoids all cross-contaminations, what is the advantage of staining the EVs by antibody staining, rather than using protein or RNA dye to ensure that all ligand binding sites are available for EV uptake. CD63 plays a role in endocytosis, and blocking its availability with an antibody might interfere with EV uptake. What is the size of a typical EV after antibody labeling; does that complex EVs together? Remeasure using ZetaView before and after? Would addition of

BSA add to the specificity of the labeling?

We appreciate this insightful comment and the discussion on labelling EVs. We agree that not all EVs from cell lines are CD63 positive. We intended to provide one example that EVs can be labeled with the EV-specific marker (i.e. antibody) to ensure the positive signals in EV-uptake assay (i.e. IF imaging) are true EV signals. We have previously performed the same EV-uptake assay utilizing a RNA selective dye (SYTO™ RNASelect™) to confirm a different labeling method (Reviewer Only Figure 2). The result suggests that the trend of EV-uptake is similar to the result of Figure 5 in the original manuscript. In addition, the size of the labeled EV with Alexa Fluor 488 conjugated CD63 antibody was measured by NTA (Reviewer Only Figure 3) and indicates that the size of labeled EVs falls within the common size range of EVs. We believe that going into extensive detail on these points may be beyond the scope of the manuscript, however, agree that some acknowledgement is necessary. We have added few sentences (L367 – L371) to address the reviewer's concern.

4. 2.1.1 Give example of "cell culture grade confocal dish"

Thank you for this suggestion. We originally meant the that the disc was a "cell-culture compatible dish consisting of a thin coverslip to facilitate confocal imaging". Any generic, compatible dish is more than acceptable for the assay. We have revised the sentences with the above language (L158 – L162).

5. 2.2.3 what is the recommended concentration of EVs to be added to recipient cells for imaging - and how long to incubate before washing off the unbound EVs?

Thank you for this question. We added the final concentration of EVs, 7.80 x 10^9 EVs, in 200 µL of media to be used in the experiment (L174). The incubation times were 4, 8, and 12 hours (L177-L178).

6. 2.2.5 apparently the cells are labeled after the EVs are added; that defeats the idea of live-cell imaging to image uptake. For the sequence described here, the imaging might as well be done with fixed cells, since all that can now be imaged is a snapshot of where the EVs are and how many of them.

This is a valid point. One purpose of this particular uptake assay is to quantify the amount of internalized EVs at certain times (4, 8, 12 hours) to investigate the level of EV uptake relative to their incubation time with living cells. We agree that the experiments can be accomplished with fixed cells. We intended to take this a step further and provide a protocol for EV uptake assay with living cells, so the protocol may be utilized for studying EV tracking and/or trafficking analysis after EV internalization. To address the reviewer's concerns, we added a note in the revised manuscript (L180 – L181).

7. 3.3 Please provide information of excitation/emission laser wavelength for CMTMR and Alexa488 and laser power settings. Higher laser power damages cells.

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. We added the information of imaging settings into the manuscript (L328 – L331).

8. 3.5 Please provide information of layer thickness in Z-stack to capture images and time laps to image EV uptake, movement through the cell, etc. Also, the characterization of EV fluorescence particles is missing. The authors must provide the EVs size analysis with frequency distribution, and define the EV fluorescence particle size.

Thank you for this question. The thickness for a Z-stack is 1 μm, and the confocal 3D image acquisition took 2 min 34 secs worth of z-stacks (each Z-plane image acquisition took approximately 8 secs; total twenty Z-stack images). We agree this information should be included in the manuscript and added detailed information (L204 – L206). In addition, The EVs size analysis with frequency distribution was added in Figure 6.

9. 4.1 What is the automatic image-processing software, and how do you use it?

The automatic image-processing software used is Imaris (Ver. 9.7.1., Oxford Instruments) and will be listed in the Table of Materials and Reagents. The detailed explanation how to use Imaris software was added into the protocol section 4. Post-imaging process (L211 – L293).

10. 4.3 What is the algorithm used to do co-localization of the two color channels?

We labeled the cellular volume with CMFDA (orange fluorescence) and EVs with Alexa Fluor 488 (green fluorescence), then built the virtual surfaces with Imaris software. The shortest distance between the EV and the cellular surface was calculated by the algorithm provided by Imaris software. The Imaris software described the function of "Spots – Shortest Distance To Surfaces" as below.

"Spots - Shortest Distance To Surfaces

Computes the shortest distance from the center of each spot of "this" Spots object to the border of all surfaces from the Surfaces objects. Distances are positive for spots outside of the Surfaces, and negative for spots inside of the Surfaces."

11. 4.4. Please provide more information (other than in the figure legend) on how the software determines cytoplasmic and membrane-bound EVs?

Thank you for this suggestion. A detailed explanation of how the software determines the internalized cytoplasmic and the membrane-bound EVs was added to the protocol section (4. Post-imaging process (L268 – L293)) to clarify these details and increase reproducibility.

Figures:
1. Figure 1: what is the principle by which the EVs are trapped in the disc? How are lipoprotein aggregates, many of which have a similar size range as EVs, eliminated?

Thank you for bringing up this question. We agree that EV isolation from lipoprotein is not straightforward. The Exodisc is a nano-filtration-microfluidics based EV isolation device., Lipoprotein or protein aggregates, sized similar to EVs, may fail to be isolated through the device.

We have added this issue to our discussion highlighting potential drawbacks to the assay (L363 – L367). To combat this concern, we adopted an EV-specific antibody (CD63) to label the EVs with a fluorescent dye, which could increase the specificity of the EVs that we used for the EV uptake assay.

2. Figures 2-4: Information of confocal microscope setup are missing, such as laser intensity, the slice interval for z-stack images, etc.

Thank you for bringing this to our attention. We have added the information of imaging settings into the figure legends (Figure 2, 3, 4).

3. Figure 2: it seems unlikely that given the numerical aperture of a 40X objective that individual EVs can be measured. Based on the scale bar, an individual pixel seems to be 250 nm, 2.5 x the diameter of an EV.

We agree that the size of EVs ranges from 40 nm to 1000 nm, and the majority of small EV size is below than the resolution limit of optical microscope. However, the fluorescently labelled EVs can be detected by the optical microscope since the enhanced fluorescence emission can be detected by the photo detector such as a camera. We added sentences to address the review's concern into the introduction part (L74 - L76) with the related reference article.

4. Figure 3: Figure 3 is taken with a 20X lens; with the average "clump of EVs" being about 1 um in diameter; what is being measured?

After the EVs internalization into the cells, EVs can be displayed as "puncta". Previous researches are also reported that the internalized EVs are looked as relatively bigger "puncta" than an individual EVs. We added the words and the references to address the reviewer's concern (L302).

5. Figure 4: according to a quick search on "split spots into surface objects" the group is using IMARIS software. It is a software to identify co-localization. According to their website, "to run this XTension the user has to create a Spots and a Surface component. For each Surface object located in the same Surpass group as the Spots object, this XTension creates a new subset of Spots that contains only the Spots that lie inside the Surface."

Yes, we used the function of "Split Spots into Surface Objects" in the previously submitted manuscript. However, we recognized that some membrane-bound EVs can also be identified as an internalized cytoplasmic EVs. Therefore, we modified the protocol and added the detailed explanation how the software determines the internalized cytoplasmic and the membrane-bound EVs (L268 – L293). We thank the review's valuable comments.

6. Figure 5: If the authors had used labeled cells to which they had added labeled EVs, they could have imaged the same cell over time to get an accurate assessment of EV uptake in individual cells. How many cells/dishes does this figure represent?

We did not capture the same cell over time, we captured the images from multiple ROIs over time.

Then, we averaged the number of internalized EVs per cell and cell volume to get Figure 5. The number of cells per each time point is approximately 150 cells.

Discussion:
1. The discussion just seems to be a summary of the results, rather than a discussion on how this technique is different from those published by others in the past. Other reports reporting 3D visualization of extracellular vesicle uptake include research articles by Durak-Kozica et al, 2018 (PMID: 30574165) or the one listed in the references Sung et al., 2020 (PMID: 32350252), but they are not critically discussed here.

Thank you for this critique. We agree that the Discussion was reiterating results, rather than introducing new ideas and critical thinking. We have updated the manuscript to incorporate a discussion on limitations of our protocol and suggested improvements (L363 – L376). Additionally, we have included information on the referenced article into the introduction section (L71 – L74).

Reviewer #3:

Manuscript Summary:

Comments on EV Uptake Manuscript.

Conceptually, is this manuscript about mechanisms to efficiently label EVs or to visualize EVs. The rationale for Exodisc was not clear until Discussion, yet based on title and introduction this was about the imaging of EV uptake. Overall, I am convinced that I would not be able to attempt this protocol even on the cell type they used as each step was not clearly stated.

We thank the reviewer for the valuable comments. We added a paragraph to depict the rationale for the use of Exodisc as a nano-filtration based microfluidic device (L64 – L68). The detailed explanation of this protocol also added in the revised manuscripts (L111 – L155).

Line 26 - I find it surprising that none of these "numerous prior" papers had sufficient methods published, so the authors may want to temper statement modestly.

We agree that the statement was bold and downplayed existing manuscripts. We have revised the statement and hope that it better reflects the improvements our protocol will hopefully bring to already established, and impressive, methods.

Line 45 - Sentence is not complete.

Thank you catching this error. We have updated the sentence (L45 – L46).

Paragraph 47 - 54. - Needs additional references and the authors should elaborate on the many different methods. This introduction is of insufficient depth to determine what has or has not been done.

We have added the discussion on the previous research about EV-uptake into the introduction (L56 - L74) and discussion section (L363 – L376).

Protocol 1.2

Exodisc is not sufficiently explained why this tool is necessary for uptake assays. If this is authors preferred choice ok, but can the imaging and analysis be used following other EV isolation methods. If so note this. What advantage does Exodisc provide over other clean up methods?

Additionally, the Exodisc Procedure is insufficiently explained - one has no idea what is going on based on this description. Is Exodisc availability?

To limit commercial bias, we have termed Exodisc a 'nano-filtration based microfluidic device'. While it is the only commercially available isolation disc of this nature, we agree that clarification is necessary to explain why we selected it for use. We have added additional information to depict the rationale for the use of a nano-filtration based microfluidic device' (L64 – L68). Additionally, we updated the explanation of the nano-filtration based microfluidic device' procedure also added into the protocol section in the revised manuscripts (L111 – L155).

1.3 "choice antibody" do you mean "antibody of choice". More importantly, how does one validate antibody for use in this procedure?

Thank you for drawing our attention to this. We hoped to make a general protocol that could be adapted to the reader's needs. While we selected CD63 to fluorescently label EVs, theoretically any validated EV specific dye would be appropriate to use. We have updated the language and added information on control experiments to Figure 1.

1.3.1 Question regarding the note: "Purpose of the experiment"? What is the rationale for using one of these dyes vs antibody conjugates. Why would one use a dye or antibody?

This is an excellent point. The antibody conjugated dye provides high specificity to visualize the EVs from other contaminants vesicles, however, the antibody may block certain antigens on the EV surface that affect the EV uptake pathways. On the contrast, lipophilic dye and RNA dye is less specific since other particle contains lipid membrane and RNA, however, these are free for antigen blocking. We added the pros and cons of the use of an antibody-conjugated dye that we used for the protocol (L367 – L371).

2.1.1 - Why is it necessary that cell types match? Is it not one of the concepts that EVs can transfer across different cell types. If you mean within species or some other aspect then you need to be specific.

Thank you for this critical note. EVs harvested in CCM originated from the adhered cells in culture. In our assay, we utilized PC3 cells. When re-introducing the harvested, labelled EVs into the uptake assay, using the same cell line (PC3) for uptake as that which the EVs were harvested from eliminates any preference between EVs and cell type. We thank the reviewer for the comment. We revised the manuscript.

2.2.1 - The explanation of what concentration of EVs to use in uptake is not explained. A number or range is needed. NTA analysis was weakly described also.

We added the final concentration of EVs, 7.80 x 10^9 EVs in 200 µL of media which was used in the experiment (L174).

2.2.3 Incubate for the desired time? What time would you recommend? Does one need to complete a time course analysis. This protocol provides no guidance on this. Why 200 ul? Why not 50 ul? Since the volume of the media in dish is unknown it is not clear why the authors have stated this number.
Thank you for noticing this missed detail. We added the recommended incubation times, 4, 8, 12 hours (L178). And the volume of media can be defined depending on the required volume of media for a certain type of dish. We also added a sentence to describe the reviewer's concern (L159 – L162).

2.2.5 What is this dye for? What prevents the continuation of internalization of EVs during this labeling.

This dye is the cytosolic staining dye for labeling the cellular area to quantify the number of internalized EVs per the volume of the cell. It is known that this dye may not affect the cellular function as the manufacturer's product description.

Steps 3 and 4. Insufficient details. I have no idea how reading this protocol will allow me to complete the experiment.
As an example Step 3.4 - Determine depth…. How?

Thank you for drawing attention to this detail. We updated Step 3.4 to explain how the depth of Z-stack was determined. We hope this adequately answers your question (L204 – L206)

Overall, I found I had numerous questions regarding each step. I might suggest that the authors write a detailed protocol for the specific cell line providing all of the details needed (time course info, actual concentrations of EVs collected, NTA values used, etc. etc. The authors can then make a statement at end of the protocol - that each type of cell will need to be independently validated. But if the readers do exactly as laid out in this protocol, on the same cell line then they should be able to achieve the same results as the authors.

We thank the reviewer for the generous comments. We revised the manuscript following the reviewer's comments.

Major Concerns:
The description is not sufficient that a reader could go do the experiment the authors are providing protocol for.

We appreciate this critical feedback. In its original state, we agree that the protocol did not contain sufficient details for reproducibility. We have revised the manuscript to hopefully address questions raised during the first iteration and by the reviewer's comments.

Reviewer #4:

Manuscript Summary:

the manuscript presents a method to visually determine whether EV are inside or attached to the surface of cells by microscopy.

Major Concerns:

it might need to describe more in detail the imaging and analyzing conditions that seem to be device-dependent

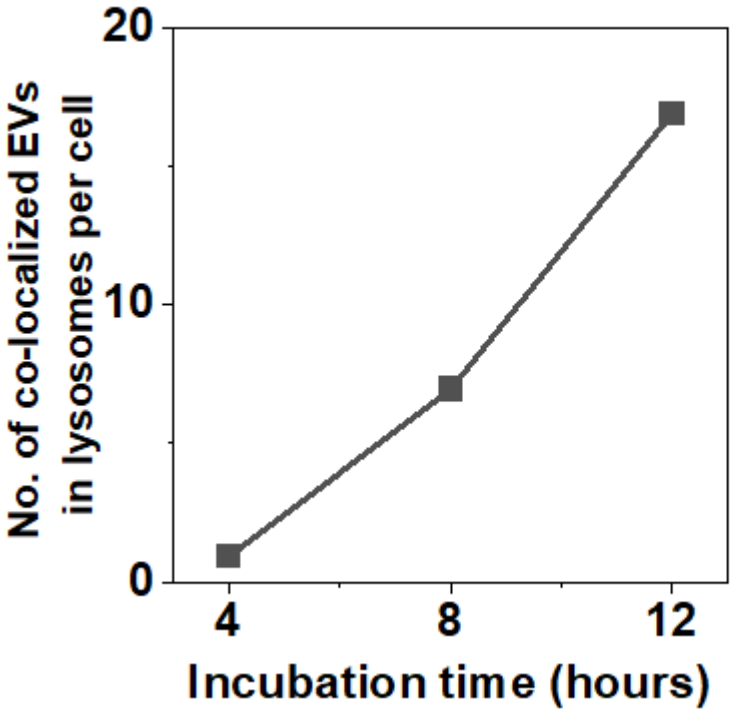
Thank you for this comment. We agree that a more detailed explanation of imaging and analysis is necessary for the reader to be able to properly use the software. We have updated the protocol to include step-by-step instructions within the software program, including, how to distinguish cell borders.

Minor Concerns:

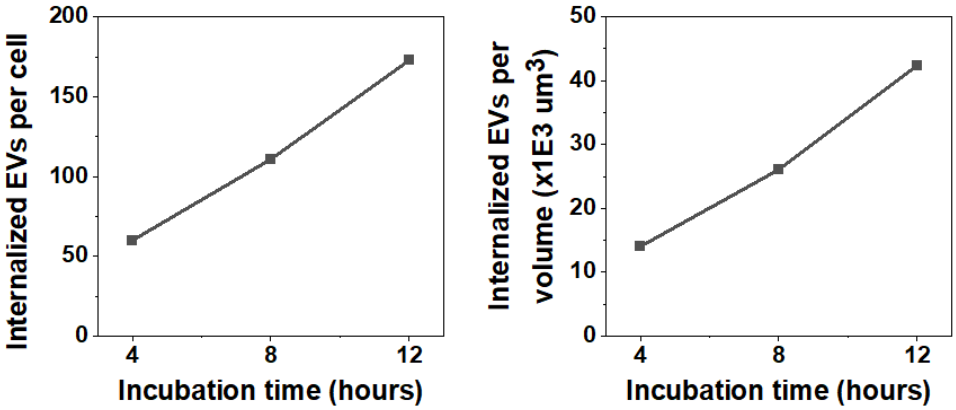
versatility towards different cell lines and EVs according to the type of protein contained

Thank you for bringing up this point. We agree that this protocol is versatile and can be applied to various cell lines. We have added several sentences to draw attention to this versatility and to encourage future work.

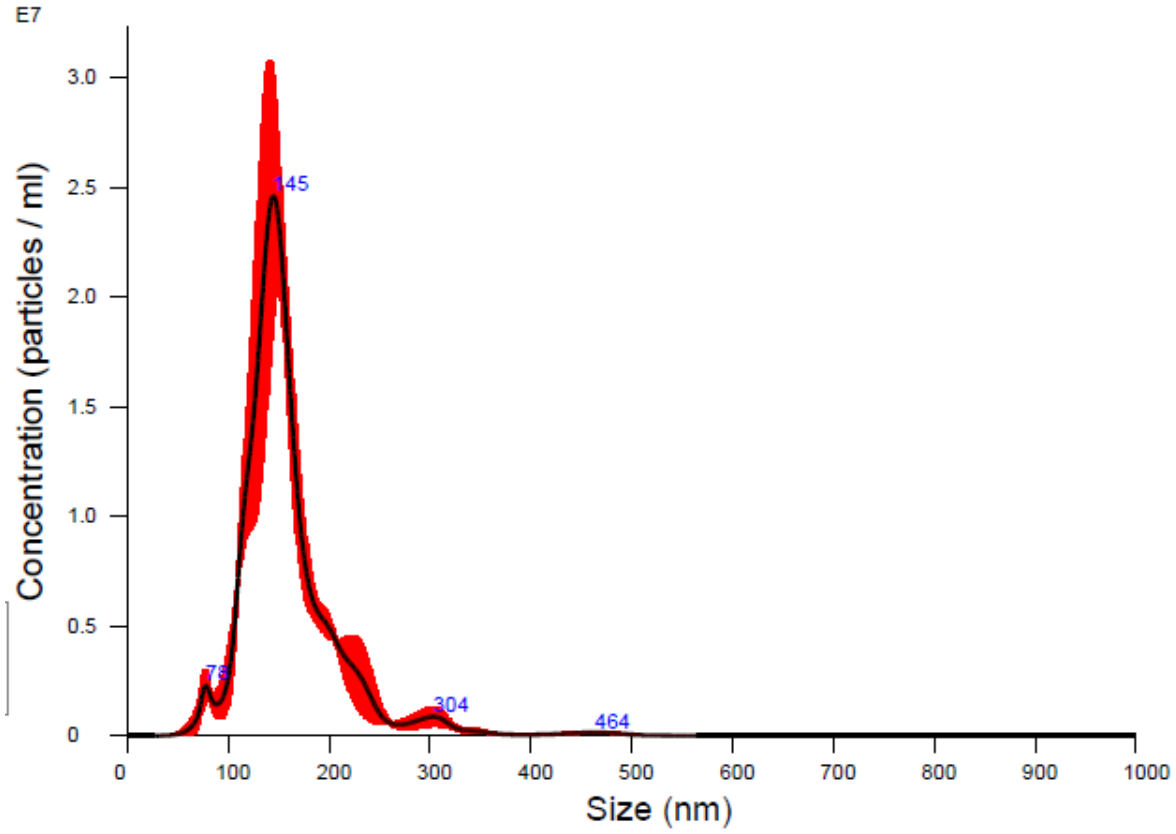
Reviewer Only Figure 1. The amount of EV co-localization as the function of incubation time.



Reviewer Only Figure 2. The amount of EV uptake depending on the duration. The EVs samples used in this experiment was labelled by RNA specific dye, SYTO™ RNASelect™.

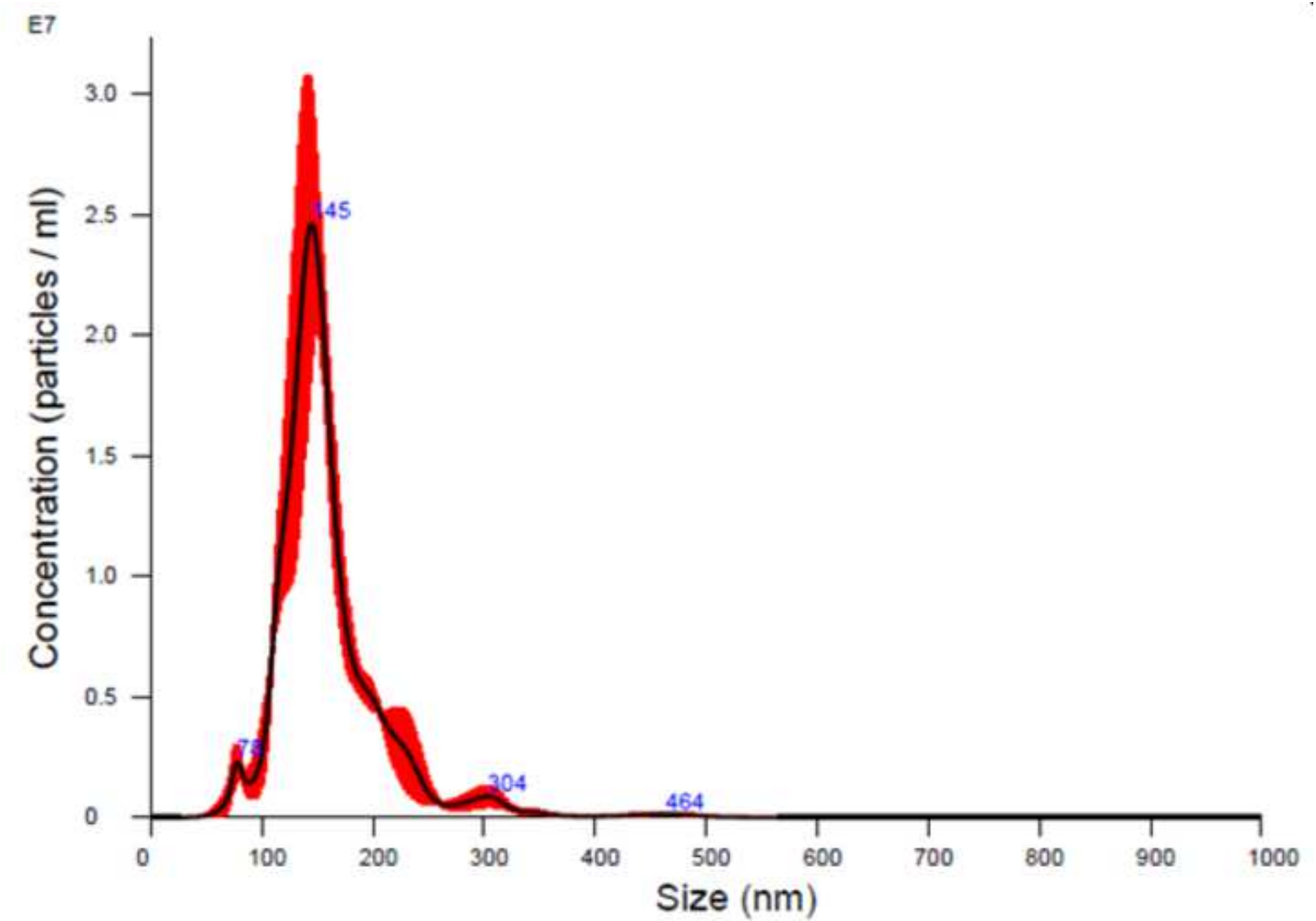


Reviewer Only Figure 3. NTA measurement of the CD63-Alexa Fluor labelled EVs



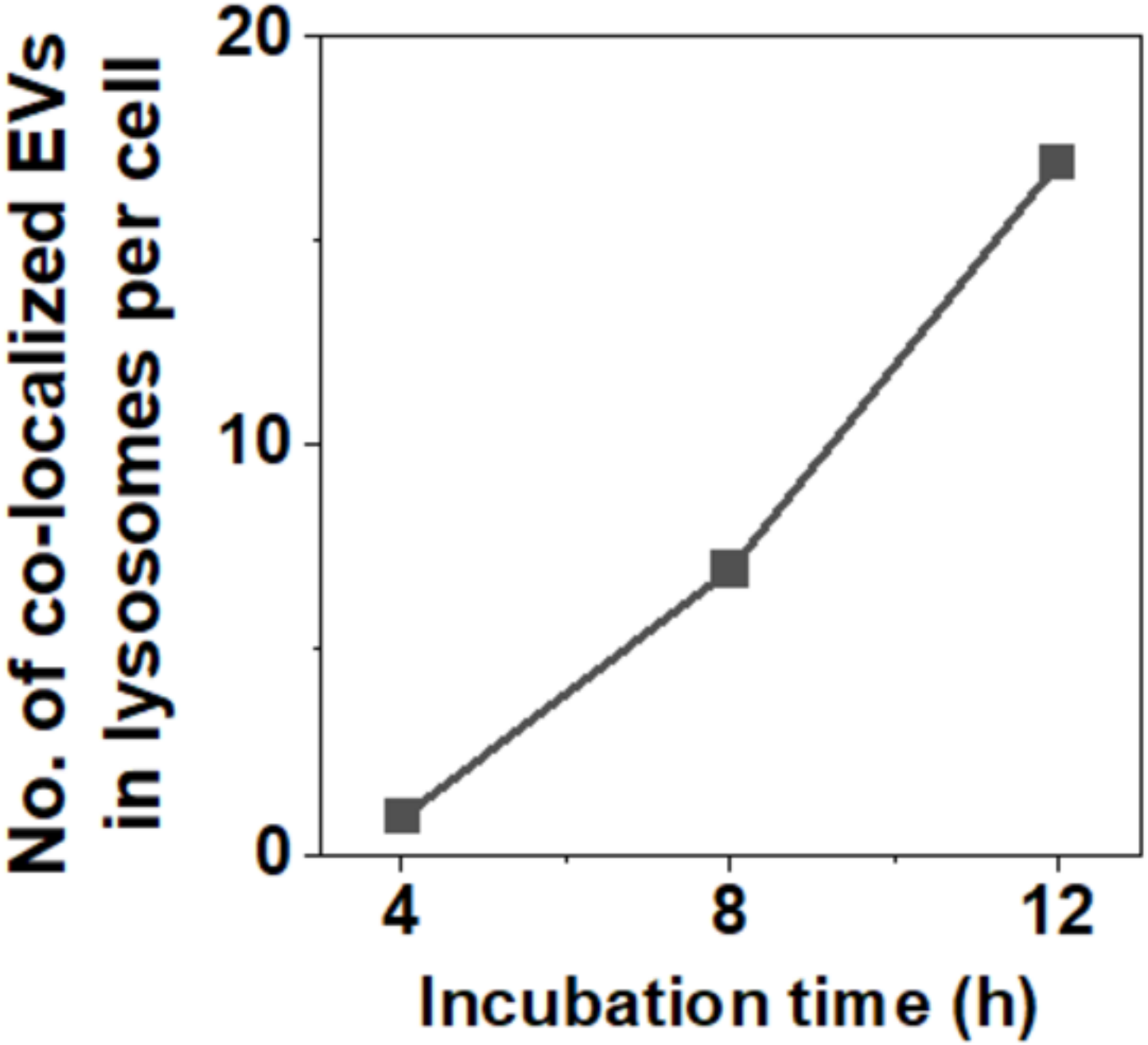
Supplementary Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);R2_Supplementary Figure 1.psd



Supplementary Figure 2

Click here to access/download;Supplemental File (Figures, Permissions, etc.);R2_Supplementary Figure 2.psd



Supplementary Figure 3

Click here to access/download;Supplemental File (Figures, Permissions, etc.);R2_Supplementary Figure 3.psd