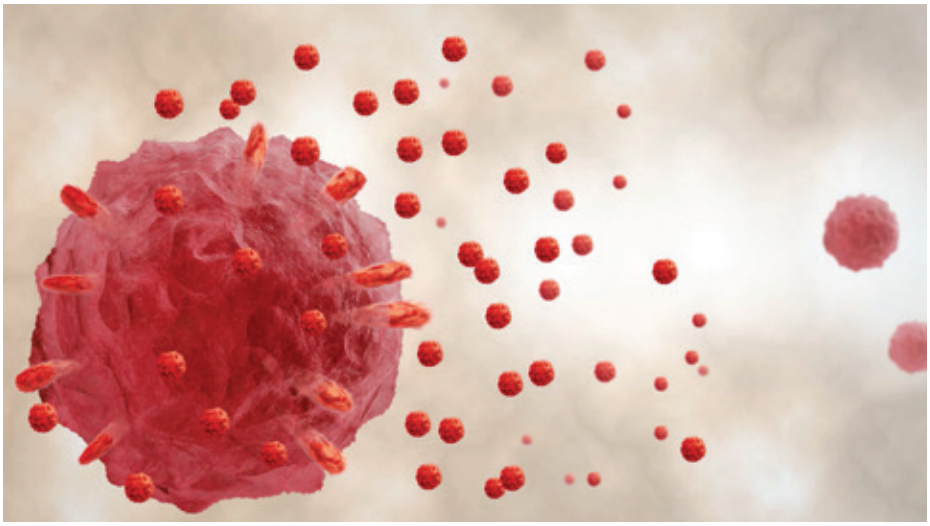
Labeling exosomal RNA and membrane components using fluorescent dyes



Introduction

Exosomes are small vesicles (30–150 nm) containing sophisticated RNA and protein cargos. They are secreted by all cell types in culture and are found to occur naturally in body fluids, including blood, saliva, urine, CSF, and breast milk [1,2]. The precise molecular mechanics for their secretion and uptake, as well as their composition, "cargo", and resulting functions, are only beginning to be unraveled. Originally thought to be just "garbage bags" used by cells to get rid of unnecessary macromolecules, exosomes are now viewed as specifically secreted vesicles that enable intercellular communication [2–4]. Exosomes have become the focus of exponentially growing interest, both to study their functions and to understand ways to use them in the development of minimally invasive diagnostics. Critical to furthering our understanding of exosomes is the development of reagents, tools, and protocols for isolation, characterization, and analysis of their RNA and protein content, as well as *in vitro* and *in vivo* tracing. A number of techniques may be used to characterize exosomes. These include the use of metabolic incorporation of modified nucleic acids (e.g., ethynyl uridine) or methionine analogs (e.g., homopropargylglycine). Such approaches may allow a more in-depth interrogation of the RNA and protein content of a given vesicle. However, simple protocols for exosomal demarcation and tracking are also very useful. Here we describe protocols for labeling exosomes with two different fluorescent dyes—one selective for RNA and the other for membrane components—that allow researchers to study the pathways and functions of exosomes in cell and animal models.

Exosome isolation

Until recently, the only option for isolation of exosomes from cell culture media and body fluids was an ultracentrifugation-based protocol [5]. Despite wide acceptance of this method in the field and its ability to recover very clean exosomes, it has a number of drawbacks. These shortcomings include extensive personnel training, a need for large amounts of starting material, low yields of exosomes, inconsistent outcomes (exosome pellets or fractions are easy to lose), processes that are highly labor-intensive and time-consuming, and lack of scalability for high throughput (a maximum of six samples at a time can be processed).

As a way to scale up, simplify, and shorten exosome isolation, Life Technologies developed the Total Exosome Isolation reagents. These enable straightforward and reliable concentration of intact exosomes from cell culture media and a variety of body fluids (serum, plasma, urine, saliva,

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CSF, milk, ascites fluid, and amniotic fluid). By tying up water molecules, the reagents force less soluble components (i.e., exosomes) out of solution, allowing them to be collected after brief, low-speed centrifugation.

Representative data are shown in Figure 1. Exosomes were extracted from HeLa cell culture media using the Total Exosome Isolation reagent, or an ultracentrifugation procedure [5] for comparison. Sizing and quantification of exosomes was performed with the NanoSight® LM10 instrument, following the manufacturer's protocol. The reagent method recovered a significant number of nanovesicles, in yields comparable to those of the ultracentrifugation procedures; all nanovesicles were smaller than 300 nm, most of them being in the typical exosome size range of 30 to 150 nm (Figures 1A, 1B).

Samples were next analyzed by western blots, using antibodies specific to CD63, a well-characterized exosomal marker [4]. Results are shown in Figure 1C, confirming that clean exosome populations were recovered with both protocols.

Next, exosomal RNA cargo was recovered with the Total Exosome RNA and Protein Isolation Kit, and the levels of five microRNAs (let7e, miR26a, miR16, miR24, and miR451) and two mRNAs (GAPDH and 18S), earlier reported to be present in exosomes [4], were analyzed by qRT-PCR. Results are displayed in Figure 1D. Based on threshold cycle (C_t) values of 25 to 33 for the majority of analytes, RNA isolation was efficient and the amount of material recovered is sufficient for standard PCR analysis. RNA recovered from exosomes derived from 30 µL of cell culture medium is sufficient for one qPCR reaction. The reagent method recovered somewhat higher levels of exosomes, compared to the ultracentrifugation procedure, as indicated by a 0.5 to 2 shift up in the C_t values for different RNAs.

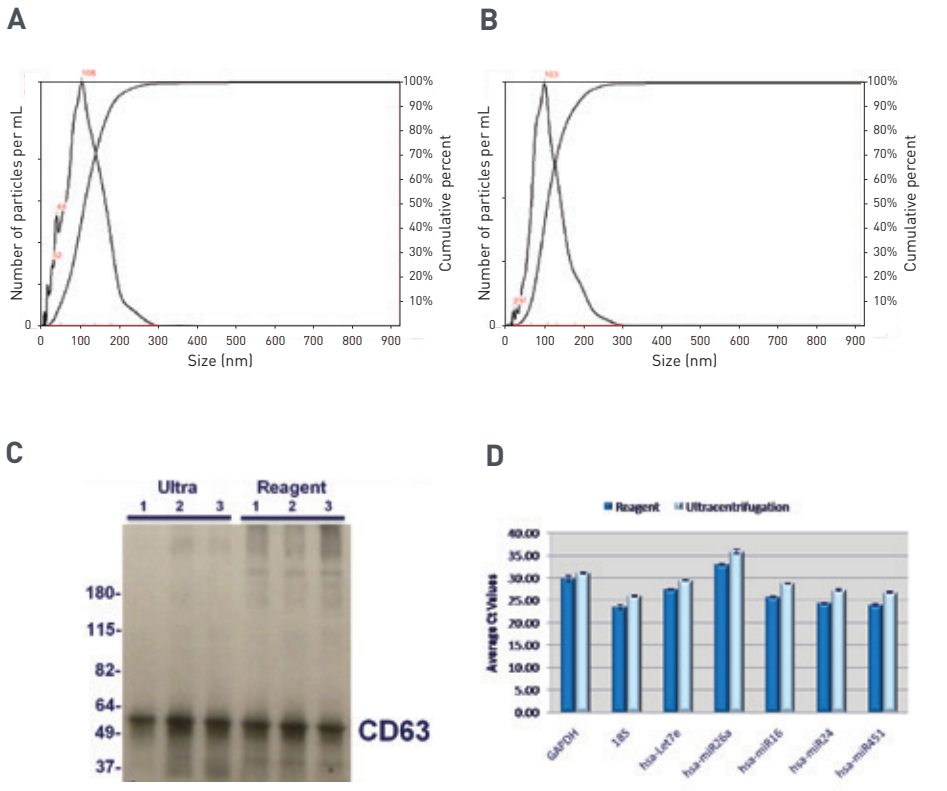

Figure 1. Exosomes isolated from HeLa cell media using Total Exosome Isolation reagents are comparable to ultracentrifugation preparations. Analysis using the NanoSight® LM10 instrument of exosomes recovered from HeLa cell culture media using **(A)** the Total Exosome Isolation reagent or **(B)** ultracentrifugation. The profiles are essentially very finely segmented histograms, indicating the number of particles per mL (in millions) for each size, binned in increments of 1 nm from 0 to 1,000 nm. **(C)** Western blot analysis for the presence of exosomal marker protein CD63 in cell culture media–derived samples. Exosomes from three separate HeLa cell culture media preparations (isolated either with the Total Exosome Isolation reagent or by ultracentrifugation) were separated on a Novex® 4–20% Tris-glycine gel under denaturing, nonreducing conditions. Standard western blot procedures with anti-CD63 antibodies were used to detect the exosomal protein marker. **(D)** Analysis of the exosomal microRNA and mRNA levels in HeLa cell culture media–derived samples by quantitative RT-PCR. RNA was isolated using the Total Exosome RNA and Protein Isolation Kit, from exosomes extracted using the Total Exosome Isolation reagent or the ultracentrifugation protocol. Levels of 5 microRNAs (let7e, miR26a, miR16, miR24, and miR451) and two mRNAs (GAPDH and 18S) were quantified by qRT-PCR, using TaqMan® assays and reagents.

Labeling of exosomal RNA and membrane using fluorescent dyes

The SYTO® RNASelect™ Green Fluorescent Cell Stain is a cell-permeant nucleic acid stain that is selective for RNA. Although virtually nonfluorescent in the absence of nucleic acids, the SYTO® RNASelect™ stain exhibits bright green fluorescence when bound to RNA (absorption/emission maxima ~490/530 nm) and only a weak fluorescent signal when bound to DNA. This stain very efficiently labels the exosomal RNA cargo [6].

BODIPY® TR ceramide is a red-fluorescent cell stain (absorption/emission maxima ~589/617 nm), which is prepared from D-*erythro*-sphingosine and therefore has the same stereochemical conformation as natural biologically active sphingolipids.

Applications of this fluorescent lipid probe include marking the structure of the Golgi complex, outlining cellular boundaries to allow observation of morphogenetic movements by confocal microscopy, tracing lipid metabolism and trafficking in living cells, and measuring rates of lipid synthesis by Schwann cells. This stain also allows very efficient labeling of the exosomal membrane [7,8]. For use in both RNA and membrane labeling, exosomes can be recovered from cell culture media or body fluids using either the ultracentrifugation protocol or the Total Exosome Isolation reagents. Two labeling approaches are described below: *in vitro* and *in vivo* labeling.

In vitro labeling of exosome RNA and/or membrane components

This protocol allows labeling of exosomes isolated from any source, including those separated from cell culture media or from any body fluid, including blood, urine, CSF, or saliva.

1. Isolate exosomes from the cell culture medium or body fluid. Use the equivalent of 100 µL of biological sample, resuspended in 100 µL of PBS, per labeling reaction.
2. Prepare a 1 mM DMSO stock solution of the dye being used (SYTO® RNASelect™ stain for RNA staining or BODIPY® TR ceramide for membrane staining).
3. Add 1 µL of the dye stock solution to the 100 µL exosome sample and mix to obtain a final dye concentration of 10 µM. Incubate at 37°C for 20 min (protect from light).
4. Remove excess unincorporated dye from the labeled exosomes with Exosome Spin Columns (MW 3,000) following the standard protocol.
5. Analyze the efficiency of exosome labeling using the Qubit® 2.0 Fluorometer (488 nm excitation for SYTO® RNASelect™ stain and 594 nm excitation for BODIPY® TR ceramide) or directly proceed with *in vivo* exosome tracing experiments, using fluorescence microscopy to detect labeled exosomes.

In vivo labeling of exosome RNA and/or membrane components

This protocol allows labeling of exosomes as they are formed within live cells. Using this method, exosomes can either be viewed and analyzed as they are secreted by cells, or after being isolated from cell media.

1. Plate cells (e.g., 2 x 10^7 HeLa cells in a T-75 flask, in 15 mL volume). Grow cells overnight in standard conditions.
2. Prepare a 1 mM DMSO stock solution of the dye being used (SYTO® RNASelect™ stain for RNA staining or BODIPY® TR ceramide for membrane staining).
3. Add 15 µL of the dye stock solution to the 15 mL of culture medium to obtain a final dye concentration of 1 µM. Incubate at 37°C for 20 min (protect from light).
4. Remove the medium, wash the cells with PBS 3 times, and add fresh medium containing exosome-depleted FBS. Culture cells for another 1–6 hr, depending on cell line and the number of exosomes required.
5. Trace exosomes as they are secreted by cells (using the FLoid® Cell Imaging Station or a fluorescence microscope), or isolate labeled exosomes from the cell medium (as previously described).

Tracing uptake of the labeled exosomes by recipient cells

This is an example of a typical study investigating the uptake of exosomes by recipient cells. Figure 2 shows the results for HeLa cells.

1. Prepare the 8-well Chamber Slide System (Thermo Fisher Scientific), with 10,000 cells/well.
2. Add the fluorescently labeled exosomes to the recipient cells.
3. Incubate at 37°C, for 30 min to 3 hr.
4. Fix cells with 4% paraformaldehyde at room temperature for 20 min.
5. Permeabilize cells with 0.1% Triton® X-100 at room temperature for 3–5 min.
6. Stain samples with Alexa Fluor® 488 phalloidin (if the exosome stain is BODIPY® TR ceramide) or Alexa Fluor® 594 phalloidin (if the exosome stain is SYTO® RNASelect™ stain) at room temperature for 20 min.
7. Mount in ProLong® Gold Antifade Reagent with DAPI.
8. Analyze samples with a fluorescence microscope.

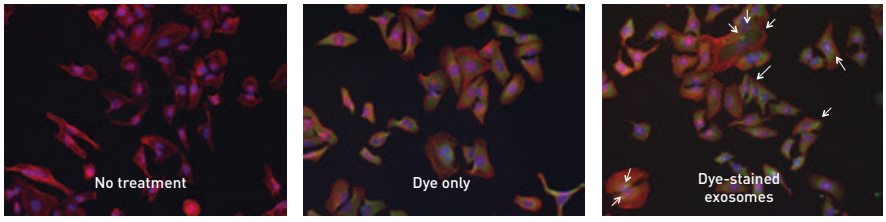

Figure 2. Uptake by HeLa cells of exosomes labeled with SYTO® RNASelect™ stain. A FLoid® Cell Imaging Station was used. Red: Alexa Fluor® 594 phalloidin; blue: DAPI; green: SYTO® RNASelect™ stain.

References

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Product	Cat. No.
SYTO® RNASelect™ Green Fluorescent Cell Stain	S32703
BODIPY® TR Ceramide	D7540
Total Exosome Isolation (from serum) (6 mL)	4478360
Total Exosome Isolation (from cell culture media) (50 mL)	4478359
Exosome Spin Columns (MW 3,000)	4484449

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