Chapter 16

Generation, Quantification, and Tracing of Metabolically Labeled Fluorescent Exosomes

Carolina Coscia, Isabella Parolini, Massimo Sanchez, Mauro Biffoni, Zaira Boussadia, Cristiana Zanetti, Maria Luisa Fiani, and Massimo Sargiacomo

Abstract

Over the last 10 years, the constant progression in exosome (Exo)-related studies highlighted the importance of these cell-derived nano-sized vesicles in cell biology and pathophysiology. Functional studies on Exo uptake and intracellular trafficking require accurate quantification to assess sufficient and/or necessary Exo particles quantum able to elicit measurable effects on target cells. We used commercially available BODIPY® fatty acid analogues to label a primary melanoma cell line (Me501) that highly and spontaneously secrete nanovesicles. Upon addition to cell culture, BODIPY fatty acids are rapidly incorporated into major phospholipid classes ultimately producing fluorescent Exo as direct result of biogenesis. Our metabolic labeling protocol produced bright fluorescent Exo that can be examined and quantified with conventional non-customized flow cytometry (FC) instruments by exploiting their fluorescent emission rather than light-scattering detection. Furthermore, our methodology permits the measurement of single Exo-associated fluorescence transfer to cells making quantitative the correlation between Exo uptake and activation of cellular processes. Thus the protocol presented here appears as an appropriate tool to who wants to investigate mechanisms of Exo functions in that it allows for direct and rapid characterization and quantification of fluorescent Exo number, intensity, size, and eventually evaluation of their kinetic of uptake/secretion in target cells.

Key words Exosomes, Metabolic labeling, BODIPY fatty acids, Differential centrifugation, Lipid analysis, Flow cytometry, Exosome uptake

1 Introduction

Since their discovery, microvesicles and exosomes (Exo) stimulated a surge of interest in the study of their biogenesis, cell targeting, and cellular effects. Exo are 50–100 nm-sized vesicles released by almost all cell types, which have been especially highlighted for their role in intercellular communication, in both physiological and pathological conditions including cancer [1, 2]. Exo biogenesis starts with the inward budding of membrane portions of late

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endosomes to form intraluminal vesicles (ILVs) that mature into multivesicular bodies (MVBs). Upon fusion with the plasma membrane, MVBs release an Exo swarm in the extracellular space that, by entering biological fluids, reaches their cell targets ultimately delivering their cargo of specific signals [3]. Despite the recent expansion of studies in the Exo field, there is a paucity of effectual methods for the reliable quantification and characterization of these vesicles. Nowadays quantification of Exo is often based on the level of total amount of proteins in vesicle preparations, but bulk protein contamination of ultracentrifuged vesicles makes quantification poorly reproducible among different preparations. A now rather diffused technology, nanoparticle tracking analysis (NTA), although promising, harbors several limitations because it has the tendency to overestimate the number of vesicles and seems less accurate for analysis of heterogeneous-sized preparations [4]. Flow cytometry, on the other hand, is a powerful technique allowing for single-particle detection and high-throughput, multiparameter analysis, but the fact that Exo vesicles have sizes below 200 nm constitutes a major challenge for the detection of small particles on the basis of their light-scattering signal, as performed in conventional FC. In recent years in the attempt to overcome such a bias, a successful new methodological approach consisting of fluorescent-labeled cell-derived vesicles coupled with high-resolution flow cytometry (hFC) analysis has been developed [4–6]. Although extremely valuable to directly analyze quantitatively and qualitatively individual cell-derived vesicles, this approach requires an optimized custom configuration of the commercially available Becton Dickinson (BD) Influx™ high-end flow cytometer that hampers its diffusion as a widely used method. Furthermore, fluorescent probes commonly used to obtain bright fluorescent vesicles preparations such as lipophilic dyes (e.g., PKH67, Di-dyes) [4] require additional washing steps to remove free unbound dye, while others, like CFSE [7], or lipid- (FM) specific dyes [8] are nonspecifically incorporated making extracellular vesicles quantification less precise. From our previous experience in labeling EVs [9], we developed a novel approach to obtain brightly fluorescent exosomes that can be examined and quantified with conventional non-customized FC instruments, i.e., Gallios (Beckman Coulter) or Canto (BD) and can be quantitatively traced in acceptor cells. Our methodology is based on cell treatment with BODIPY®-labeled fatty acid analogues that upon uptake enter the cellular lipid metabolism ultimately producing fluorescent Exo as a direct result of biogenesis. The great advantage of using fluorescent fatty acid analogues is that they enter the cellular lipid metabolic pathway without affecting the natural lipid metabolism or perturb the lipid homeostasis inside the cell. Nascent Exo, once released in the cell medium, can be routinely isolated by well-established differential centrifugation protocols (Fig. 1a) that can be coupled with density gradient floatation (Fig. 1b). Purified fluorescent Exo

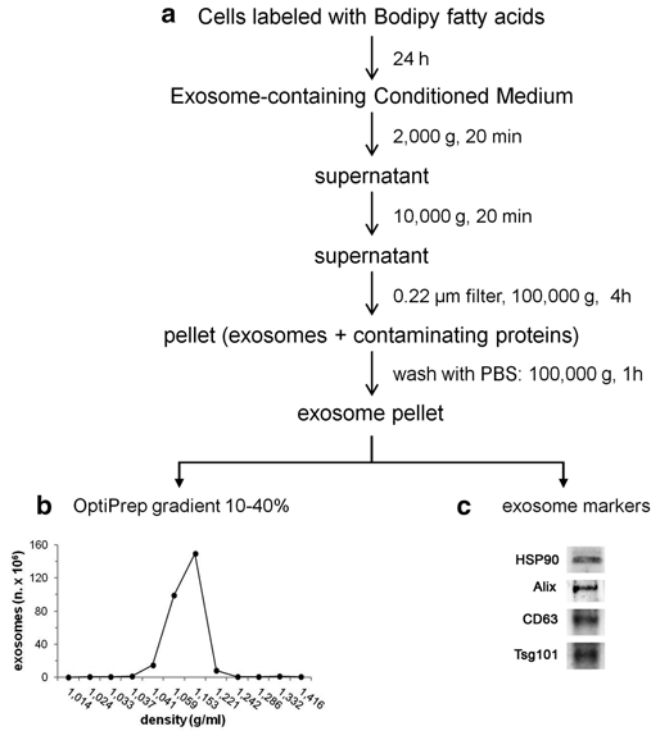

Fig. 1 Exosome purification. (**a**) The experimental workflow used for fluorescent Exo isolation based on differential ultracentrifugation. Exo-containing conditioned medium from BODIPY fatty acid-labeled cells is processed by differential centrifugation to remove intact cells and cell debris. Resuspended Exo plus contaminating proteins pellet is filtered using a 0.22 μm membrane filter before last centrifugation step. The speed and length of each centrifugation are indicated. The final Exo pellet is resuspended in PBS and can be either directly FC counted or (**b**) further purified by running overnight on an 10–40 % OptiPrep density gradient. The fluorescent peak displays a density ranging from 1.06 to 1.15 g/mL typical of Exo [10, 11]. (**c**) Western blot analysis of purified exosomes (5×10^7) probed with antibodies against Exo markers HSP90, Alix, CD63, and Tsg101

display a density ranging from 1.06 to 1.15 g/mL typical of Exo [10, 11] and are positive for Exo markers (Alix, Tsg101, CD63, HSP90) (Fig. 1c). We used both green fluorescent hexadecanoic acid (BODIPY FL C_{16}) (C16) and red fluorescent dodecanoic acid (BODIPY 558/568 C_{12}) (C12) that are reported to incorporate well into cells [12, 13] but as far as we know never to label MVBs. The characteristics that highlight BODIPY-conjugated lipids as close to natural membrane molecules cannot be extended to synthetic lipophilic fluorescent probes so far largely introduced for Exo research, especially when they are used to trace membrane trafficking of Exo following cell uptake. Metabolic labeling of Exo is thus an effective tool that may consent precise quantification of the natural extent and timing of Exo production related to cell cycle, type, and culture conditions. Furthermore our methodology

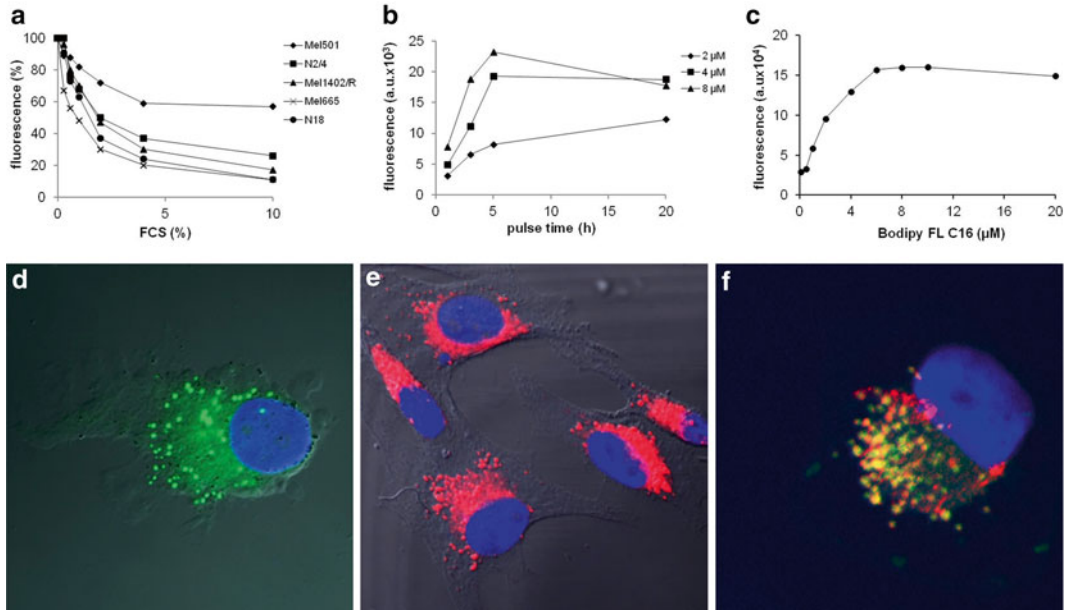

Fig. 2 Metabolic labeling of Me501 cells with BODIPY fatty acids. (**a**) FCS dependence of C16 incorporation in different cell lines. Cell-associated fluorescence is analyzed by FC. In all cell lines tested 0.3% FCS ensures optimal C16 cell incorporation. (**b**) Time dependence of C16 incorporation into Me501 cells. Cell-associated fluorescence is maximal after 5 h incubation with all C16 concentrations tested. (**c**) C16 concentration dependence of Me501 cell incorporation. After 5 h incorporation, a plateau is reached at 7 μM probe concentration. (**d**, **e**) Confocal microscopy cross sections of Me501 cells metabolically labeled for 5 h with (**d**) C16 or (**e**) C12 showing fluorescence localization in the ER/late endosomal/MVB compartments. (**f**) Colocalization of C16 with anti-bis(monoacylglycero)phosphate (BMP) antibody, an Exo-specific lipid [14]. As expected, BMP is completely absent on plasma membrane and consequently from microvesicles budding from the plasma membrane (ectosomes)

enables the measuring of single Exo-associated fluorescence transfer to cells, thus making quantitative the correlation between Exo uptake and activation of cellular processes (Fig. 2).

In conclusion we can infer that metabolically labeled vesicles positive for Exo markers (HSP90/Alix/CD63/Tsg101) are likely to represent a real Exo population that can be characterized and measured as a single particle by means of affordable FC instruments available to a wider scientific community.

2 Materials

2.1 Cell Culture

1. Human melanoma Me501 cells.
2. Cell culture medium: RPMI 1640, complete with required nutrients and antibiotics (e.g., L-glutamine, penicillin/streptomycin) and supplemented with 10% (v/v) fetal calf serum (FCS) heat inactivated and filtered through a 0.22 μm filter.
3. Tissue culture flasks.

2.2 Cell Labeling with BODIPY Fatty Acid Analogues

1. Green fluorescent fatty acid: BODIPY FL C_{16} (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-hexadecanoic acid) (C16). Resuspend powder in methanol at 1 mM final concentration. Make 100 µL aliquots in Eppendorf tubes and store at –20 °C (up to 12 months) (*see* **Note 1**).
2. Red fluorescent fatty acid: BODIPY 558/568 C_{12} (4,4-difluor o-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid) (C12). Resuspend powder in methanol at 1 mM final concentration. Make 100 µL aliquots in Eppendorf tubes and store at –20 °C (up to 12 months).
3. Cell-labeling medium: cell culture medium, 0.3% FCS (*see* **Note 2**).
4. 2 mL round bottom tube (Eppendorf) (*see* **Note 3**).
5. Bovine serum albumin (BSA) essentially fatty acid-free 2% in PBS (*see* **Note 4**).
6. KOH 20 mM.
7. Phosphate-buffered saline (PBS).
8. Microfuge.
9. ThermoMixer (Eppendorf).

2.3 Exo Isolation

1. Exo production medium: RPMI 1640 supplemented with all the nutrients and antibiotics and 10% exosome-depleted FBS (Exo-FBS) (*see* **Notes 5** and **6**).
2. 50 mL conical tubes (Falcon).
3. Beckman ultracentrifuge.
4. SW41 Ti Rotor, Swinging Bucket (Beckman Coulter).
5. SW60 Ti Rotor, Swinging Bucket (Beckman Coulter).
6. Polyallomer konical™ tubes appropriate for the ultracentrifuge SW41 Ti Rotor and SW60 Ti Rotor (Beckman Coulter) (*see* **Note 7**).
7. Sterile PBS.
8. Stock OptiPrep™ solution: 60% (w/v) aqueous iodixanol.

2.4 Analysis of Fluorescent Lipids

1. *Lipid extraction*: extraction solvent: chloroform/methanol 2:1 (v/v) [15].
2. Double-distilled water (ddH_2O).
3. 15 mL polypropylene centrifuge tubes or glass tubes.
4. Vortex.
5. Centrifuge.
6. N_2 tank.
7. *TLC analysis*: silica gel plates with fluorescence indicator.

8. Solvent mixture for separation of neutral lipids (*see* **Notes 8** and **9**): hexane/diethyl ether/acetic acid (70:30:1, v/v).
9. Solvent mixture for separation of phospholipids (*see* **Note 9**): chloroform/methanol/32% ammonia (65:35:5, v/v) [16].
10. Lipid stain mixture: 3% copper acetate and 8% ortofosforic acid. Make 100 mL in a glass container.
11. Neutral lipid standards: triglyceride, cholesterol, and cholesterol ester. Make a pool in chloroform/methanol 2:1 (v/v) containing 20 μg of triglycerides and 5/10 μg each of cholesterol, cholesterol ester in 20 μL.
12. Phospholipid standards: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), sphingomyelin (SM), cardiolipin (CL), and bis(monoacylglycerol)phosphate (BMP). Make a pool in chloroform/methanol 2:1 (v/v) containing 20 μg of each standard in 20 μL.
13. Filter paper.
14. Glass syringe (Hamilton).
15. TLC glass tank.
16. Glass tray of suitable size to accommodate TLC plate.
17. Oven.
18. Typhoon Phosphorimager for fluorescence detection (GE Healthcare).
19. Scanner.

2.5 Protein Quantitation (Bradford Assay)

1. BSA standard stock (2 mg/mL).
2. Protein Assay Dye Reagent Concentrate (Bio-Rad).
3. Test tubes.
4. PBS.
5. Cell lysis buffer: 10 mM Tris–HCl pH 7.4, 150 mM NaCl, 60 mM octylglucosyde, 5 mM EDTA, 1% Triton, Protease Inhibitor Cocktail Tablet, 1 in 10 mL (Sigma) (*see* **Note 10**).
6. Plastic or quartz cuvettes.
7. Spectrophotometer.

2.6 Western Blot Analysis

1. Blotting System (Bio-Rad) and transfer membranes (Hybond C Extra, GE Healthcare Life Sciences).
2. TBST: 10 mM Tris–HCl pH 8, 150 mM NaCl, 0.1% (w/v) Tween 20.
3. Blotto: 5% (w/v) skim milk powder in TBST.
4. Transfer buffer: 20 mM Tris–HCl, pH 7.4, 150 mM glycine, 20% methanol.

5. Running buffer: 25 mM Tris–HCl, pH 7.4, 192 mM glycine, 0.1 % SDS.
6. Ponceau S staining solution: 0.5 % (w/v) Ponceau S, 1 % acetic acid.
7. Mouse anti-TSG101mAb (Santa Cruz): 1:200 in Blotto.
8. Rabbit polyclonal anti-CD63 Ab (System Bioscience): 1:1000 in Blotto.
9. Mouse anti-Hsp90 mAb (Santa Cruz):1:200 in Blotto.
10. Mouse anti-Alix mAb (Abcam):1:1000 in Blotto.
11. HRP conjugated secondary antibody 1:3000 in TBST.
12. ECL SuperSignal West Pico Chemiluminescent Substrate (PIERCE).
13. FluorChem™ Q System (Protein Simple).

2.7 Flow Cytometry (FC)

1. Size and number calibration beads: green fluorescent (505/515) Flow Cytometry Submicron Particle Size Reference Kit (Life Technologies).
2. Exo counting beads: Flow-Count Fluorospheres (Beckman Coulter).
3. PBS filtered through 0.22 μm membrane filter.
4. 5 mL polypropylene round bottom tubes (Beckman Coulter).
5. Gallios Flow Cytometer and Kaluza Software (Beckman Coulter) or similar.

2.8 Fluo-Exo Cell Transfer Assay

1. HBSS (Hank's Balanced Salt Solution), 20 mM HEPES.
2. ToPro-3 (viability dye): make 1 mM stock in DMSO (*see* **Note 11**).
3. 96 well plates.
4. Orbital shaker.
5. 5 mL polypropylene round bottom tubes (Beckman Coulter).
6. Fluorescence intensity calibration beads: Quantum™ FITC-5 MESF (Molecules of Equivalent Soluble Fluorophores) (Bangs Laboratories, Inc.).

3 Methods

3.1 BODIPY Fatty Acid Preparation

1. Thaw an aliquot of C16 or C12 (*see* **Note 12**).
2. Place the tube with lid open under a fume hood and evaporate off the solvent under a stream of N2 at room temperature (RT).
3. Add 30 μL KOH, 20 mM (*see* **Note 13**).
4. Vortex until complete solubilization.

5. Spin 20 s at 12,100 × *g* with microfuge.
6. Put tube in ThermoMixer at 60 °C for 10 min (*see* **Note 14**).
7. Add 70 µL BSA 2% in PBS (v/w) and mix by pipetting (*see* **Note 15**).

3.2 Cell Labeling with BODIPY Fatty Acids

1. Adherent cells (Me501) are cultured to 50% confluency in cell culture medium in 75 cm^2 flasks at 37 °C with 5% CO_2 (*see* **Note 16**).
2. Remove medium and wash with 5 mL PBS.
3. Add 4 mL of cell-labeling medium containing 7 µM BODIPY fatty acids (*see* **Note 17**).
4. Incubate 5 h in at 37 °C with 5% CO_2 (*see* **Note 17**).
5. Remove medium, wash cells twice with PBS to eliminate probe in excess, add 36 mL/flask of Exo production medium, and return cells to the incubator for 24 h (*see* **Note 18**).
6. Collect cell culture supernatant (exosome-containing conditioned medium, ECM) and proceed with Exo isolation or keep at 4 °C (*see* **Note 19**).

3.3 Fluorescent Exo Isolation

1. Transfer ECM to a 50 mL centrifuge tube.
2. Centrifuge 20 min at 2000 × *g*, 4 °C and discard pellet.
3. Transfer supernatant to 12 mL ultracentrifuge tubes (for SW41 Ti Rotor) (*see* **Note 20**).
4. Centrifuge 20 min at 10,000 × *g*, 4 °C and discard pellet.
5. Filter supernatant with 0.22 µm membrane filter and place in a fresh 12 mL polyallomer tube (SW41 Ti).
6. Centrifuge 4 h at 100,000 × *g* (*see* **Note 21**).
7. Discard supernatant and add PBS to fill the tube (*see* **Note 22**).
8. Centrifuge 1 h at 100,000 × *g*.
9. Discard supernatant and resuspend Exo pellet with 100 µL of PBS (*see* **Notes 23** and **24**).
10. For a further step of purification, perform an OptiPrep™ (iodixanol) gradient separation as follows: add Exo (260 µL in PBS) to 1 mL of 60% OptiPrep™.
11. Prepare a discontinuous gradient: 60% (w/v), 40% (w/v), 30% (w/v), and 10% (w/v) solutions of OptiPrep™ by diluting 60% stock with PBS.
12. In a 4.5 mL tube suitable for *SW60* Ti Rotor, place at the bottom Exo diluted in OptiPrep™ and then gently lay 0.5 mL 40% OptiPrep™, 0.5 mL 30% OptiPrep™, and 1.8 mL 10% OptiPrep™.
13. Centrifuge 18 h at 192,000 × *g*.

14. Collect 12 × 330 µL fractions and FC count as described in Subheading 3.7 (*see* **Note 25**).
15. To determine the density of a fraction, run in parallel a control OptiPrep™ gradient. Collect fractions as described above and measure the refractive index on 5 µL of each fraction with a refractometer (*see* **Note 26**).

3.4 Analysis of Fluorescent Lipids

1. *Lipid extraction*: transfer Exo or cells in PBS to a 15 mL tube and add ddH_2O to reach 1.5 mL final volume.
2. Add 6 mL chloroform/methanol 2:1 (v/v) (*see* **Note 27**).
3. Vortex and centrifuge 20 min at 2,240 × *g* at RT.
4. Remove aqueous upper phase (containing non-lipid cellular material) and interface.
5. Evaporate the lower chloroform phase under a gentle nitrogen stream at 50 °C. It takes about 20 min.
6. Add to each tube a suitable volume of chloroform/methanol 2:1 (v/v) in order to have the equivalent of $5–10 \times 10^7$ Exo or 5×10^5 cells in 20 µL (*see* **Notes 28** and **29**).
7. *TLC analysis*: mark lanes needed for samples and standards with a fine point pencil and a ruler, making the loading mark at least 1 cm from the bottom of the plate (*see* **Note 30**).
8. Using a glass syringe, spot or streak 20 µL of the lipid sample and pooled standards. Allow sample to dry.
9. For neutral lipid separation (Fig. 3a/d): line the TLC tank with filter paper and pour 30 mL of solvent mixture. Put the lid on the tank and let the solvent equilibrate for at least 30 min (*see* **Note 31**). When ready to run samples, pour 10 mL of fresh solvent mixture in a glass tray that can accommodate the TLC plate and place at the bottom of the TLC tank.
10. For phospholipid separation (Fig. 3e/f): pour 100 mL of fresh solvent mixture in the TLC tank.
11. Lower the loaded TLC plate into the chromatography tank making sure the samples are above the surface of the developing solvent (*see* **Notes 32** and **33**).
12. Place the lid on the tank and allow the solvent to ascend to about 1 cm from the top of the TLC plate (*see* **Note 34**).
13. Remove plate from the tank and air-dry in a fume hood (*see* **Note 35**).
14. Scan plates with a Typhoon Phosphorimager system.
15. Quantify the fluorescence intensity of the lipid bands using available software (ImageQuant).
16. After quantification of fluorescent bands, nonfluorescent lipid standards can be visualized by immersing the TLC plate for 10 min in a glass tray containing 100 mL of staining solution.

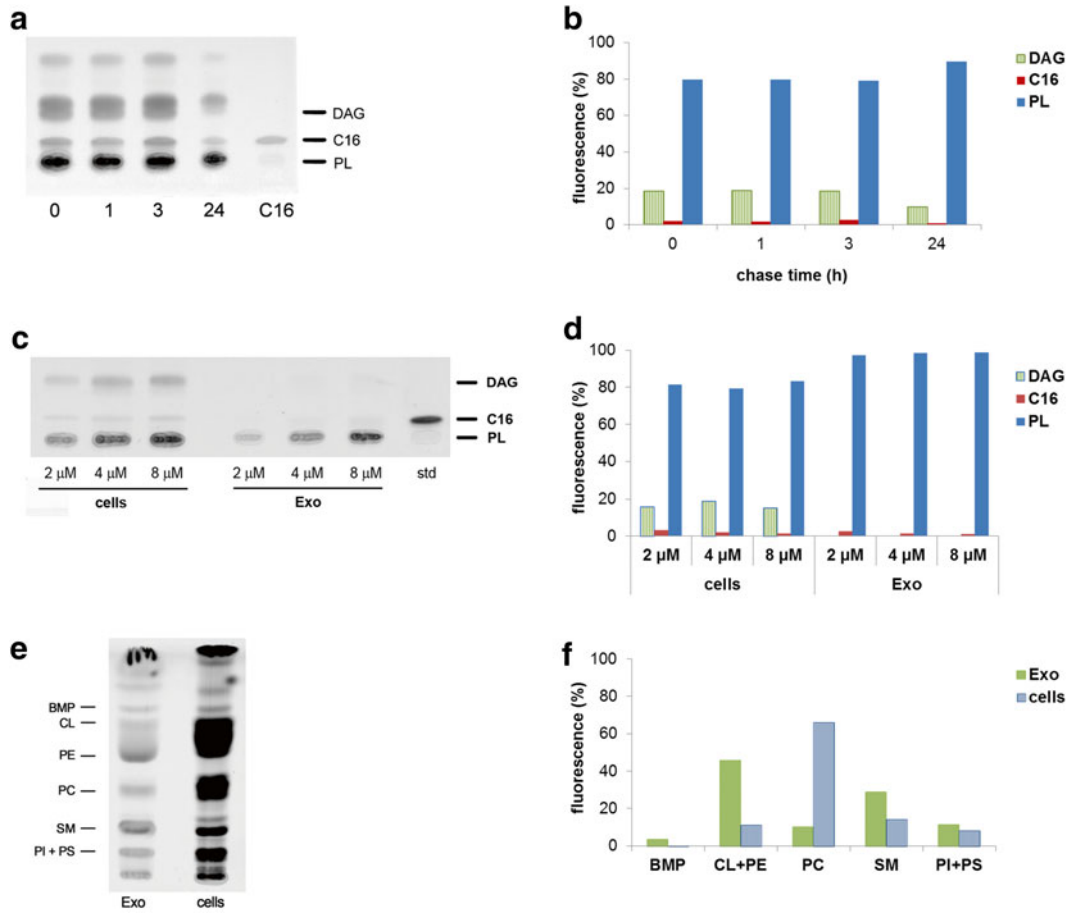

Fig. 3 Lipid analysis of C16-labeled cells and Exo. (**a**) Cells were treated for 5 h with 7 µM C16 and chased in complete media. At different time points, lipids were extracted and subjected to TLC analysis for neutral lipids. (**b**) Quantification of relative density of lipid spots shows that at the end of the incubation time, most fluorescent probe is incorporated into phospholipids/DAG and only very little is still present as free probe as determined by comparing cells and Exo. (**c**) Cells were treated with different concentrations of C16 and after 24 h cells and Exo were collected and analyzed by TLC for neutral lipids. (**d**) Quantification of relative density of lipid spots shows that in contrast to cells, Exo contain mostly phospholipids and virtually no free C16. (**e**) Cells and Exo lipid extracts are analyzed by TLC for phospholipids showing that C16 is metabolized in all the major phospholipid classes including Exo-specific BMP whose relative ratio is much higher in Exo if compared to cells. (**f**) Quantification of relative ratio of lipid spots shows that differences in relative amounts of phospholipids between Exo and cells are representative of their unlabelled counterpart [14] with the exception of BMP which is greatly enriched in Exo. Abbreviations used: *SM* sphingomyelin, *CL* cardiolipin, *PS* phosphatidylserine, *PI* phosphatidylinositol, *PE* phosphatidylethanolamine, *PC* phosphatidylcholine, *BMP* bis(monoacylglycero) phosphate

17. Remove the plate, air-dry (*see* **Note 36**), and bake in preheated oven for 10 min at 120 °C.
18. Scan image and analyze with Alphaview or other software.

3.5 Protein Quantitation (Bradford Assay)

1. Prepare a duplicate set of BSA dilutions, starting with 40 μg/mL and performing twofold dilutions in dH_2O (six dilutions). 800 μL for each dilution will be needed.
2. Prepare a blank reference standard not containing protein (800 μL of dH_2O).
3. Add cell lysate (*see* **Note 37**), Exo, or OptiPrep™ fractions (*see* **Note 38**), to 800 μL of dH_2O (final volume) (*see* **Note 39**).
4. Add 200 μL of Protein Assay Dye Reagent Concentrate.
5. Vortex and transfer samples and standards into cuvettes.
6. Read OD at 590 nm within 10 min.

3.6 Western Blot Analysis

1. For each lane, prepare a tube with 5×10^7 exosomes (counted as described in Subheading 3.7) or ~20 μg cell lysate (~5×10^4 cells) (*see* **Note 37**).
2. Add SDS sample buffer to each tube and heat for 5 min at 95 °C (*see* **Note 40**).
3. Load samples on a 10% or 12% gel.
4. Perform electrophoresis at constant 160 V for 1 h 30 min.
5. Following electrophoresis, electro-transfer proteins onto nitrocellulose membranes using the Blotting System.
6. At the end of the run, check the quality of the protein transfer by incubating the nitrocellulose membrane for 1–2 min in Ponceau S solution (*see* **Note 41**).
7. Move the membrane to a clean tray and wash with dH_2O until red protein bands become visible.
8. Acquire the image of Ponceau stained membrane by a densitometer or scanner.
9. Block membranes with Blotto for 1 h at RT. Care should be taken not to touch and disrupt the membrane.
10. Probe the membranes with primary antibodies (i.e., anti-HSP90, anti-Alix, anti-TSG101, anti-CD63) O/N in Blotto at 4 °C (*see* **Notes 42** and **43**). Wash membrane three times in TTBS for 10 min.
11. Incubate membrane with HRP-labeled secondary antibody for 1 h at RT. Wash membrane three times in TTBS for 10 min.
12. Prepare the ECL working solution of the SuperSignal West Pico Chemiluminescent Substrate (*see* **Note 44**) by mixing Reagent 1 and Reagent 2 at 1:1 ratio (v/v) (*see* **Note 45**). Incubate the membrane in the ECL working solution for 60 s.
13. Visualize the protein bands using FluorChem™ Q System (Protein Simple).

3.7 FC Analysis of Fluo-Exo

Although it is well known that FC based on light-scattering detection of vesicles and particles smaller than 300 nm is severely hampered by noise derived from buffers, optics, and electronics, we present here a protocol apt to discriminate fluorescently labeled vesicles from nonfluorescent noise by applying fluorescence threshold triggering. With this setup and by using control 100–500 nm fluorescent beads, we can distinguish Exo from noise events on the basis of fluorescence. One hundred nanometer beads of known number are also used as internal reference standard in each FC acquisition. Numerous repeated acquisitions of different dilutions of beads show linearity and reproducibility of measurements demonstrating that quantification of fluorescent Exo is accurate (Fig. 4):

1. Set FL1 discriminator to 1 on histogram and FL1/LogSS dot plot in order to fix the threshold on fluorescence intensity just above the PBS background noise (*see* **Note 46**).
2. Acquire PBS samples and increase FL1 voltage until PBS background noise is barely visualized.
3. Add 20 μL to Flow-Count Fluorospheres (*see* **Note 47**) to 200 μL of PBS. Set instrument at flux high; fix the stopping gate on 2000 Flow-Count Fluorospheres on correctly drawn region in a FL channel different from FL1, e.g., FL5 (Fig. 4c) (*see* **Note 48**). Modify FL1 voltage (*see* **Note 49**) in order to count no more than 150 events of background noise in respect to 2000 Flow-Count Fluorospheres (*see* **Note 50**).
4. Add 100,000 of each 0.1, 0.2, 0.5, and 1.0 μm sizes and number calibration beads to 200 μL of PBS in separated tubes.
5. Acquire at least 5000 diluted beads to set the mean fluorescence of the different-sized beads (Figs. 4a and b). Analyze the samples by plotting fluorescence at 525/40 nm (FL1) versus log scale side scatter (LogSS). The level of fluorescence threshold will exclude the PBS background noise only (Fig. 4a, histogram and dot plot) but will allow a clear identification of the smaller beads of 0.1 and 0.2 μm sizes (Fig. 4a, histogram and dot plot) (*see* **Note 51**).
6. *FC exosome count*: prepare sample by mixing 5 μL of Exo resuspended in PBS with 20 μL of Flow-Count Fluorospheres (*see* **Notes 47** and **52**) in 200 μL PBS final volume and a blank tube containing PBS as control of background noise.
7. Set instrument at flux high, fix the stopping gate on 2000 Flow-Count Fluorospheres on previously drawn (Subheading 3.7, **step 3**) region in a FL channel different from FL1, e.g., FL5 (Fig. 4c) (*see* **Note 48**), and register the events in the exosome region correctly drawn in FL1 (Fig. 4d) (*see* **Note 53**).

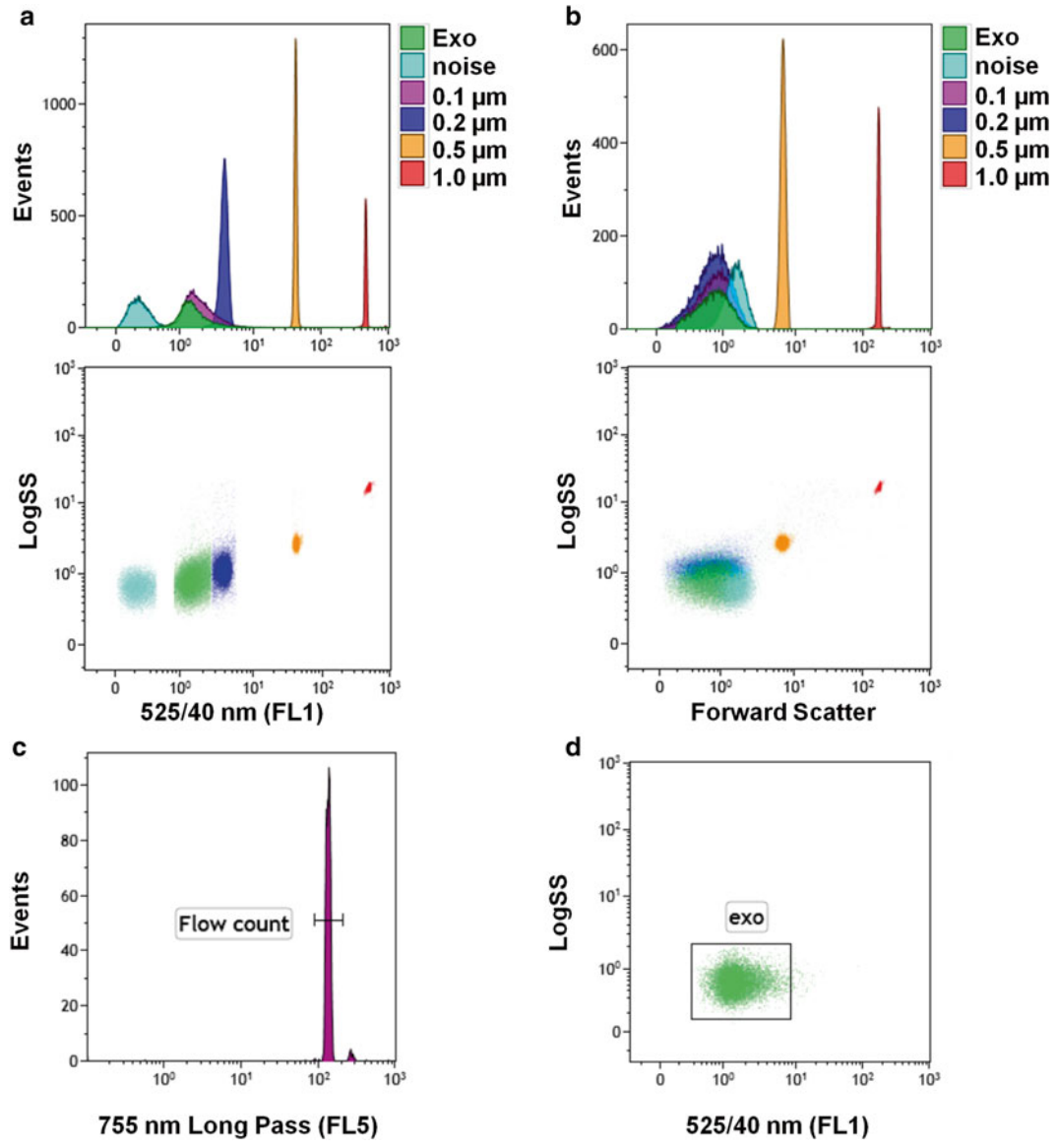

Fig. 4 Flow cytometry analysis of C16-Exo. C16-Exo, fluorescent beads ranging in size from 0.1 to 1.0 μm and background noise (noise) were analyzed for fluorescence (**a**, 525/40 nm FL1) and size (**b**, forward scatter). For more clarity, in these histograms and dot plots, the noise signal is shown although in the analyses it should not appear as described in Subheading 3.7. The pair color/sample of the legend matches with sample colors reported in the histograms (*upper side*) and dot plots (*lower side*) of (**a**) and (**b**). Two thousand Flow-Count Fluorospheres were used to determine Exo number. Exo sample was resuspended in PBS with Flow-Count Fluorospheres. The instrument was set to fix the stopping gate on 2000 Flow-Count Fluorospheres on correctly designed linear region in FL5 channel (**c**), while the number of Exo was registered in the rectangular Exo region previously drawn in FL1 (**d**)

8. To determine if there is a correspondence between the number as indicated on the package of 0.1 μm size standard fluorescent beads and the bead number counted by FC acquisition, prepare a tube with 10^7 beads of 0.1 μm size standard fluorescent with 20 μL of Flow-Count beads (*see* **Note 47**) in 200 μL PBS and follow the instructions reported in step 4 (*see* **Note 54**).
9. Total Exo and size standard fluorescent beads number can be established according to the formula: $x = ((y \times a/b)/c) \times d$ where y = events counted at 2000 counting beads; a = number of counting beads in the sample; b = number of counting beads registered (2000); c = volume of sample analyzed; and d = total volume of exosome preparation.

3.8 Fluo-Exo Cell Transfer Assay

1. The day before the experiment, seed 5×10^4 cells per well in a 96 well plate in duplicate for each Exo concentration (0–500 Exo per cell) plus one well for cell counting (*see* **Note 55**).
2. Gently remove media from cells and wash once with PBS.
3. To each well add 100 μL HBBS, 20 mM HEPES containing different amounts of Exo. To a well add 100 μL HBBS, 20 mM HEPES without Exo to test for autofluorescence.
4. Incubate 4 h at 37 °C with gentle shaking in an incubator equipped with an orbital shaker.
5. Remove medium, wash once with PBS, and add about 100 μL PBS.
6. Detach cells by gently pipetting and transfer to a fresh tube. Add PBS to reach 200 μL final volume (*see* **Note 56**).
7. Dead cells are excluded from analysis by adding ToPro-3 to ~20 nM final concentration just prior to FC analysis (*see* **Note 57**).
8. *Quantification of Exo and cells associated fluorescence* (Fig. 5): to quantify both Exo and cell-associated Exo, prepare a Quantum™ FITC-5 MESF standard curve by setting up five tubes with 50 μL PBS and three drops (~120 μL) of each bead with different amounts of fluorescein plus a blank tube with only PBS. Prepare a tube with Exo in PBS.
9. Acquire Exo, cell samples, and Quantum™ FITC-5 MESF beads with FC and determine arithmetic means for each sample.
10. To determine MESF per Exo and cells, transform fluorescence data (arithmetic mean) of Exo using the QuickCal analysis template provided with each Quantum™ MESF lot.
11. Transform MESF associated to cells in number of Exo transferred by using the formula: transferred Exo number = [cell fluorescence (MESF) − autofluorescence (MESF)]/MESF associated to a single Exo.

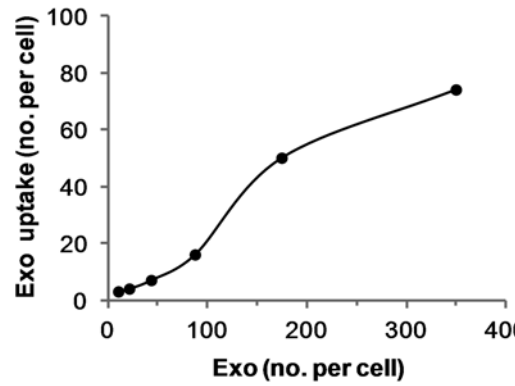

Fig. 5 Analysis of Exo transfer to cells. Me501 cells were incubated for 4 h with the indicated concentrations of fluorescent Exo. Single Exo-associated fluorescence is determined as described in Subheading 3.8, **step 8**. Flow cytometry analysis shows dose-dependent uptake of exosomes

4 Notes

1. When handling fluorescent probes, perform all the operations in the dark.
2. The amount of BODIPY fatty acid cell incorporation is dependent on FCS concentration in culture media and varies with cell type (Fig. 2a). The FCS concentration that gives the best uptake of the fluorescent probe should be determined prior to labeling a new cell type.
3. With this type of tube, we had better recovery of probe from tube walls.
4. BODIPY fatty acids are complexed to BSA to avoid lipotoxicity induced by free fatty acids. BSA must be fatty acid-free for optimal binding with BODIPY fatty acids [17].
5. In our hands Exo-FBS has proven to give the best recovery of fluorescent Exos in terms of Exo number.
6. Some batches of Exo-FBS may present turbidity; in this case centrifuge 5 min at 560 × *g* and filter through 0.45 μm membrane filter.
7. If sterility is required, use sterile centrifuge and ultracentrifuge tubes and perform all steps in a tissue culture hood.
8. For separation of neutral lipids, TLC tank must be presaturated with the solvent mixture prior to immerse TLC plates as described in Subheading 3.4, **step 9**.
9. Prepare 100 mL solution and keep in an airtight container until ready to use. It can be reutilized in future experiments.
10. The same lysis buffer is used for Western blot analysis of cells.

11. Store at 4 °C. Prior to use make a 1:1000 dilution in PBS (50× stock).
12. It has to be noted that for Exo quantification experiments, we have only used C16 since the FC laser excitation required for C12 (ranging from 558 to 568 nm) has been difficult to achieve with common instruments equipped with 488 nm argon-ion laser resulting in poor and unreproducible Exo quantification. On the other hand, metabolic labeling of Exo with C12 can be successfully applied in experiments where Exo are tracked by other means, i.e., confocal microscopy, in vivo imaging, or FC equipped with optional lasers.
13. KOH is necessary to avoid micelle formation and to solubilize fatty acids in aqueous solutions.
14. If BODIPY fatty acid is not completely solubilized, after 5 min in ThermoMixer, vortex vigorously and extend incubation at 60 °C for additional 5 min.
15. It can be prepared in advance and stored at −20 °C. It can be frozen and thawed multiple times.
16. Cells must be in exponential growth rate when labeled with BODIPY fatty acids to allow for maximal probe incorporation.
17. Add 28 μL of 1 mM stock BODIPY fatty acid to 4 mL of medium. In Me501, a primary melanoma cell line, we obtained optimal fluorescence cell incorporation after 5 h incubation with C16 (Fig. 2b) at a probe concentration of 7 μM (Fig. 2c). C16 concentration and time of incubation may vary with different cell types. It has also to be noted that when a new cell type is used, several control experiments should be performed to ensure that fluorescent fatty acid analogues are metabolized as would be expected for native fatty acids. In particular they should include proof of esterification into phospholipids and neutral lipids by TLC and absence of unesterified C16 (Subheading 2.4) (Fig. 3a/f). Fluorescence intensity of Exo should also be checked to make sure they still can be FC counted (Subheading 3.7) .
18. The volume of medium during the step of Exo secretion is critical for an abundant Exo recovery. 24–36 mL is optimal for a 75 cm^2 flask.
19. Can be stored at 4 °C up to a week.
20. It is only necessary to use sterile equipment if the final use of exosomes is going to require sterility. Otherwise very clean, but not necessarily sterile, tubes are required.
21. Best recovery of fluorescent Exo can be obtained by ultracentrifugation for 4 h.

22. This step is required to remove proteins in excess that could disturb Exo FC counting and western blot analysis.
23. Exo in PBS can be stored up to 2 days at 4 °C before FC counting.
24. Store Exo at −20 °C. Avoid repeated freezing and thawing and check again Exo number after thawing.
25. Iodixanol up to 60% (5 μL in 200 μL PBS) does not interfere with FC counting and protein determination by the Bradford method.
26. As an alternative measure the absorbance (optical density) of the fractions as described [18].
27. Up to 10^6 cells and 1×10^{10} Exo can be extracted with this volume of solvent.
28. This is the minimal amount of lipids to load in each lane to obtain a good resolution.
29. Tightly closed samples can be stored at 4 °C until needed.
30. Lanes must be 1 cm wide and distant 1 cm from each other.
31. Presaturation of the tank is an essential step to obtain a good separation of neutral lipid species.
32. Sample spots should remain above the surface of the developing solvent; otherwise samples could leach in the solvent.
33. For neutral lipid separation, the bottom edge of the TLC plate must be put in the glass tray.
34. Separation usually takes 10–15 min for neutral lipids and 30–40 min for phospholipid separation.
35. Plates can also be quickly dried with a hair dryer.
36. After baking fluorescent bands are not visible anymore on TLC plates.
37. Prepare cell lysates as follows: to $1–2 \times 10^6$ cell pellet, add 300 μL of lysis buffer and incubate 30 min at 4 °C on ice, centrifuge 5 min at $2000 \times g$, and discard pellet. Use 2 μL of lysate for protein determination. Prepare lysis buffer as described in Subheading 2.5, **step 5**.
38. Forty times of dilutions of 60% iodixanol (5 μL in 200 μL PBS) do not interfere with protein determination by the Bradford method.
39. The color changes immediately. If the color of some samples is obviously out of line with the BSA standard curve, prepare new tubes immediately with more or less sample.
40. Some antibodies against Exo markers, e.g., CD63 and CD81, give better resolution if sample buffer does not contain 2-mercaptoethanol or dithiothreitol (DTT).

41. Ponceau S allows reversible staining of total proteins blotted on nitrocellulose membranes.
42. All antibody incubations are carried out using gentle orbital shaking.
43. For a complete list of Exo marker antibodies, check 10.
44. If no protein band is visible, use ECL SuperSignal West Dura (PIERCE) for higher sensitivity.
45. Two milliliters per membrane is sufficient.
46. PBS must be filtered through a 0.22 μm filter which is used to dissolve the EV samples. Be careful to resuspend exosome population with a 0.22 μm-filtered PBS to remove eventual precipitates that could be mistakenly registered as events. Before running samples check instrument performance following the procedures suggested by the manufacturer.
47. Corresponding to 19,020 beads, but it depends on the batch.
48. On the Gallios instrument used to set up this protocol, it corresponds to 520 V.
49. Corresponding to about 2–3 events per second, in the presence of more events per seconds, follow the cleaning procedures suggested by the manufacturer; otherwise if the cleaning procedures fail, the PBS preparation must be verified.
50. This step is necessary to bypass the lack of exosome visualization by using a threshold set on size-based forward scatter. In fact the FS of background noise, 0.1 and 0.2 μm, are indistinguishable (Fig. 4b, histogram and dot plot).
51. To establish the right amount of Exo to be counted for each cell type/culture condition, make multiple dilutions of Exo in PBS to verify linearity and choose the right dilution for further measurements.
52. Each Fluorosphere contains a dye which has a fluorescent emission range from 525 to 700 nm when excited at 488 nm.
53. Verify that Exo population (Figs. 4a and b, green histograms and dots) lies below the 0.2 μm size in terms of fluorescence intensity (Figs. 4a and b, blue histograms and dots) but above the excluded background noise (Figs. 4a and b, turquoise histograms and dots).
54. To check for precision of instrument and reproducibility of measurements, it is necessary to determine if 0.1 μm beads counted at different dilutions display linearity.
55. Just prior to the transfer assay, count cells in a well to determine cell number per well.
56. Strongly adherent cells may require a trypsin/EDTA and centrifugation step before being resuspended in PBS.
57. Use 4 μL of 50× stock in 200 μL.

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