bio-protocol

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A Robust Nanoparticle-based Magnetic Separation Method for Intact Lysosomes

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Abstract

Lysosome isolation is a preresiquite for identifying lysosomal protein composition by mass spectroscopic analysis, to reveal lysosome functions, and their involvement in some diseases. Magnetic nanoparticle-based fractionation has received great attention for lysosome isolation, owing to its high efficiency, purity, and preservation of lysosomal structures. Understanding the intracellular trafficking of magnetic probes is the key point of this technique, to determine the appropriate time for magnetic isolation of lysosomes, because this parameter changes depending on different cell lines used. The traditional magnetic probes, such as superparamagnetic iron oxide nanoparticles (SPIONs), require surface modification by fluorescent dyes to enable the investigation of their intracellular trafficking, which has some disadvantages, including the possible alternation of their bio-interaction, and the instability of fluorescence properties in the lysosomal environment. To overcome those limitations, we present a protocol that employs magnetic-plasmonic nanoparticles (MPNPs) to investigate intracellular trafficking using their intrinsic imaging capability, followed by quick lysosome isolation using a magnetic column. This protocol can be easily applied to isolate the intact lysosomes of any adherent cell lines.

Keywords: Lysosomes, Nanoparticles, Magnetic separation, Plasmonic imaging, Endocytosis, Endolysosomal pathway, Intracellular trafficking

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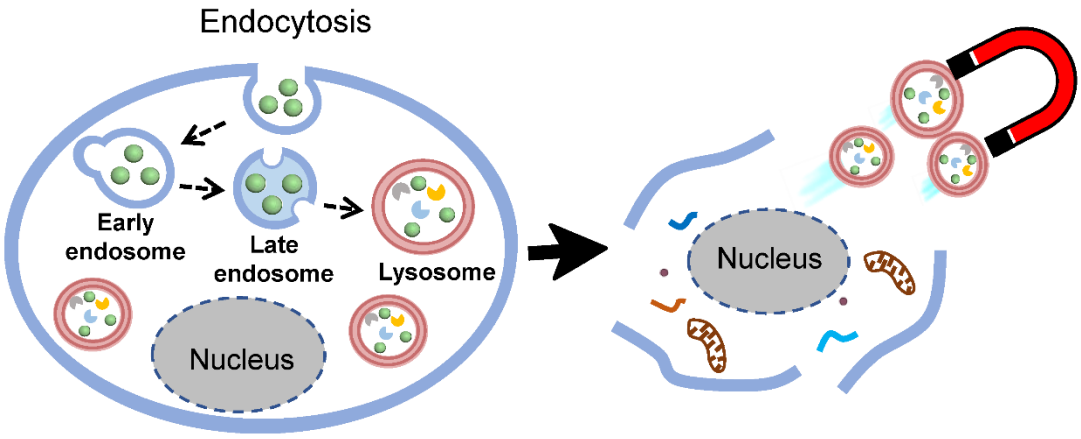
Graphical abstract:



Background

Since their discovery by Christian de Duve in the 1950s (De Duve *et al.*, 1955), the role of lysosomes in cellular function has been explored extensively, which led to the change of the view of lysosomes from a static digestive system, to the dynamic regulator of cellular metabolism. As indicated in various studies, lysosomal dysfunctions are found to be linked with the group of metabolic disorders known as lysosomal storage diseases (Mukherjee *et al.*, 2019). Therefore, understanding lysosomal biology in both normal and pathogenic conditions is crucial to figuring out the mechanistic insights of lysosomal activity, to facilitate diagnostic methods, or establish a new therapeutic strategy.

The rapid and efficient isolation of lysosomes is a prerequisite to identify lysosomal protein composition, using proteomic analysis to reveal their involvement in cellular functions or disease progression. So far, several strategies have been developed to isolate lysosomes, including density-gradient centrifugation, immunoaffinity purification, and magnetic nanoparticle-based fractionation. Among these approaches, a nanoparticle-based method that delivers magnetic nanoparticles to the lumen of lysosomes, through an endocytic pathway, followed by a separation process, using a magnetic column, has been proven to be able to isolate lysosomes with the highest yield and purity, while efficiently preserving their integrity (Singh *et al.*, 2020).

The accurate understanding of intracellular trafficking of magnetic nanoparticles is a key step to prevent contamination by other organelles (*i.e.*, endosomes) in the magnetic nanoparticles-based fractionation of lysosomes. Generally, SPIONs are used as magnetic probes, which generally requires employing fluorescent dye-based techniques to monitor their intracellular trafficking. However, it has been suggested that the lysosomal environment could lead to quenching and/or distortion of fluorescence dye signals, which may cause an ensuing effect on the interpretation of the data (Milosevic *et al.*, 2017). In addition, the surface modification of nanoparticles with dye molecules may influence the nano-bio interactions, which results in the alteration of the cellular uptake and intracellular trafficking of nanoparticles (Snipstad *et al.*, 2017; Thomsen *et al.*, 2021). Herein, to further refine the magnetic nanoparticle-based fractionation, the magnetic-plasmonic Ag/FeCo/Ag core/shell/shell nanoparticles (MPNPs) are used as multifunctional probes for lysosome isolation. Owing to their plasmonic properties, the intracellular trafficking of MPNPs can be easily investigated using confocal laser scanning microscopy, to confirm the accumulation of MPNPs in lysosomes, prior to magnetic isolation.

This protocol outlines the optimized procedures for preparation of MPNPs, intracellular trafficking study of MPNPs, and magnetic isolation of lysosomes. The time required for completing magnetic isolation of lysosomes after cell homogenization is within 30 min, which is significantly shorter than that of the density-gradient centrifugation technique. The amount of protein obtained was sufficient for mass spectroscopy, to identify protein

composition. More importantly, this protocol was demonstrated to be easily adaptable to other adherent cell lines (Le *et al.*, 2022).

Materials and Reagents

1. Glass syringe with lock tip 2 mL (Cadence Science, Stock Keeping Unit: 2407)
2. Glass syringe with lock tip 5 mL (Cadence Science, Stock Keeping Unit: 2417)
3. Stainless steel 304 syringe needle, noncoring point 2 inch 12G (Sigma-Aldrich, catalog number: Z116947-1EA)
4. Stainless steel 304 syringe needle, noncoring point 6 inch 20G (Sigma-Aldrich, catalog number: Z102709-1EA)
5. Centrifuge tube 50 mL (AS One, catalog number: 2-3939-03)
6. Microtube 1.5 mL (AS One, L-2057)
7. VIOLAMO 5 mL tube (AS One, catalog number: 2-4118-01)
8. Centrifuge tube 15 mL (AS One, catalog number: 1-3500-21)
9. Round cover glass *Φ*12mm No.1 (Matsunami, catalog number: C012001)
10. White slide glass edge grinding S1111 (AS One, catalog number: 2-154-01)
11. Terumo syringe with needle 2.5 mL 23G blue (AS One, catalog number: 1-2044-03)
12. Parafilm membrane (Amcor, Parafilm M, catalog number: PM996)
13. CELLect® Fetal bovine serum, 500 mL (FBS; MP Biomedicals, catalog number: 2917354H)
14. High-purity Ar gas, >99.9999 vol.%
15. Cobalt (II) acetylacetonate, 97% (Co precusor; Sigma-Aldrich, catalog number: 227129-50G)
16. Iron (III) acetylacetonate, 99.99% (Fe precusor; Sigma-Aldrich, catalog number: 517003-50G)
17. Silver nitrate, 99.9999% (Ag precusor; Sigma-Aldrich, catalog number: 204390-10G)
18. 1,2-hexadecanediol, 90% (Sigma-Aldrich, catalog number: 213748-50G)
19. Oleylamine, 70% (Sigma-Aldrich, catalog number: O7805-500G), stored at 4°C
20. Oleic acid, 90% (Sigma-Aldrich, catalog number: 364525-1L), stored at 4°C
21. Tetraethylene glycol (Sigma-Aldrich, catalog number: 110175-1KG)
22. Acetone, 99.5% (Kanto Chemical, catalog number: 01026-70)
23. Hexane, 96% (Kanto Chemical, catalog number: 18041-70)
24. Chloroform, 99% (Kanto Chemical, catalog number: 07278-70)
25. Toluene, 99% (Wako Pure Chemical, catalog number: 201-01871)
26. 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-350] (PEG350-DOPE; Avanti, catalog number: 880430O-25MG), stored at −20°C
27. 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine-*N*-(glutaryl) (18:1 Glutaryl PE; Avanti, catalog number: 870242C-25MG), stored at -20°C
28. 2-morpholinoethanesulfonic acid, monohydrate, (MES; Dojindo, catalog number: 341-01622)
29. N-hydroxysuccinimide (NHS; Thermo Fisher Scientific, catalog number: 24500), stored at 4°C
30. Ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC; Dojindo, catalog number: 346-03632), stored at 4°C
31. Amino dextran, MW. 10,000 (aDxt; Thermo Fisher Scientific, catalog number: D1860), stored at 4°C
32. Dulbecco's phosphate buffer (PBS; Nissui Pharmaceutical, catalog number: 05913), stored at 4°C
33. Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, catalog number: 08456-36), stored at 4°C
34. COS-1 cells (available from American Type Culture Collection, catalog number: CRL-1650)
35. Poly-*L*-lysine (PLL) solution, 0.01% (Sigma-Aldrich, catalog number: P4832-50ML)
36. 4%-paraformaldehyde phosphate buffer, 500 mL (PFA; Nacalai Tesque, catalog number: 09154-85), stored at 4°C
37. Digitonin (Wako Pure Chemical, catalog number: 043-21376), stored at 4°C
38. Ammonium chloride (NH_4Cl, Wako Pure Chemical, catalog number: 015-02991)
39. Bovine serum albumin (BSA; Sigma-Aldrich, catalog number: A8022-50G), stored at 4°C
40. Alexa Fluor® 647 mouse anti-human CD107A (AF647@CD107A; BD Biosciences, catalog number: 562622), stored at 4°C

41. 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific, catalog number: D1306), stored at 4°C
42. VECTASHIELD® Antifade mounting medium (Vector Laboratories, catalog number: H-1700), stored at 4°C
43. MES buffer (0.1 M, pH ~6) (see Recipes)
44. PBS buffer (see Recipes)
45. Digitonin solution (see Recipes)
46. Ammonium chloride solution (see Recipes)
47. DAPI staining solution (see Recipes)
48. Protease inhibitor solution (PIS) (see Recipes)

Note: The specific storage temperatures are indicated. Otherwise, chemicals are stored at room temperature (RT).

Equipment

1. Analytical balance (Sartorius, model: ME253P)
2. Three-neck round bottom flask 50 mL with angled side necks, center joint: ST/NS 29/42, side joints: ST/NS 15/25 (Tokyo Garasu Kikai, catalog number: 371-13-06-01)
3. Strong magnetic stirrer oval *Φ*12 × 25 mm (AS One, catalog number: 4-2687-04)
4. Laboran screw tube bottle 13.5 mL (glass vials; AS One, catalog number: 9-852-06)
5. Liebig condenser 300 mm, bottom joint: 29/42, top joint: 19/38 (Tokyo Garasu Kikai, catalog number: 330-15-51-14)
6. Digital high accuracy temperature controller (AS One, TJA-550, catalog number: 1-6124-01)
7. Mantle heater 50 mL (Tokyo Technological Labo, model: S-05)
8. High power stirrer (AS One, HPS-100, catalog number: 1-4136-01)
9. Flowmeter (Kofloc, model: RK1250)
10. Septum rubber, white, natural, for 18 mm tube (FUJIFILM Wako Pure Chemical, catalog number: 195-11771, Japanese Article Number: 4987481378957)
11. Fisherbrand™ Pasteur pipets (Fisher Scientific, catalog number: 22-063156)
12. Double element thermocouple WK-*Φ*3.2×200 (AS One, catalog number: 3-9391-14)
13. Trap sphere, top and bottom joints: 29/42 (Tokyo Garasu Kikai, catalog number: 330-15-91-07)
14. Refrigerated centrifuge (Kubota, model: 5910 (with RS-410M rotor))
15. Ultraviolet-visible absorption spectrophotometer (JASCO, model: V-750)
16. Two-neck round bottom flask 50 mL with an angled side neck, center joint, and side joints: 14/24
17. TS one-neck round bottom flask 100 mL,15/25, with the glass stopper (Climbing Co., ltd., CL0070-05-11)
18. Sonicator (AS One, Ultrasonic Cleaner ASU-6, oscillation frequency: 40 kHz)
19. High-speed micro centrifuge (Hitachi Koki, model: Himac CF15RXII (with T16A31 rotor))
20. Ultracentrifuge (Eppendorf Himac Technologies, model: CS100FNX (with S100AT4-2004 rotor))
21. 37°C and 5% CO_2 incubator (ESPEC, model: BNA-111)
22. Confocal laser scanning microscope (CLSM; Olympus, model: FV1000D)
23. Cell Lifter (Corning, product number: 3008)
24. MidiMACS separator starting kits (Miltenyi Biotec, catalog number: 130-042-301)
25. MS Column (Miltenyi Biotec, catalog number: 130-042-201)
26. High-speed refrigerated micro centrifuge [Tomy Seiko, model: MDX-310 (with AR015-24 rotor)]

Software

1. Fiji (NIH/https://imagej.net/software/fiji/), with color clustering and coloc 2 plugins

Procedure

A. Preparation of MPNPs, by a combination of a polyol, and a one-pot synthesis

Note: The details on the formation mechanism of MPNPs using this method can be found in Takahashi et al. (2015).

The glassware for synthesizing MPNPs were shown in Figure 1.

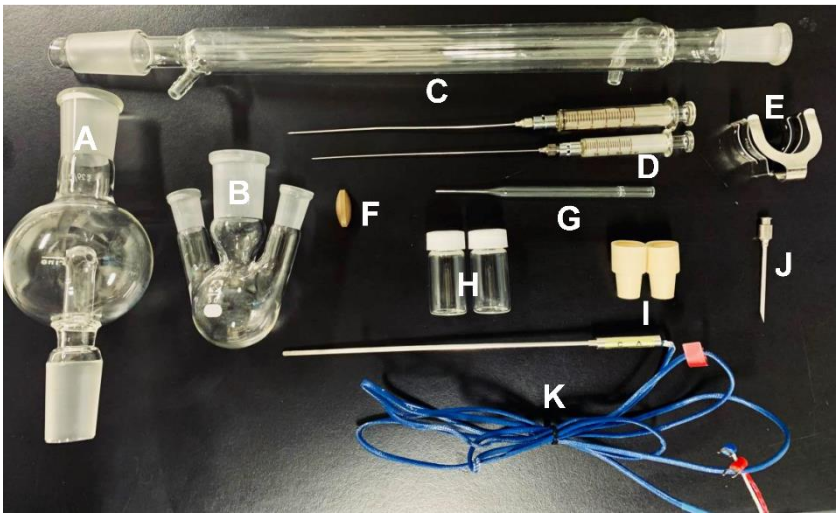


Figure 1. Glassware for preparation of MPNPs.
(A) Trap sphere, (B) Three-neck round bottom flask, (C) Liebig type reflux condenser, (D) Glass syringes with needles, (E) Clamp, (F) Oval magnetic stir bar, (G) Pasteur pipette, (H) Glass vials, (I) Septum rubbers, (J) Needle, and (K) Thermocouple.

1. Weigh 0.1 mmol of silver nitrate, and 1.0 mmol of 1,2-hexadecanediol.
2. Place an oval magnetic stir bar in the three-neck round bottom flask, and transfer the weighed silver nitrate followed by 1,2-hexadecanediol into the flask. Then, sequentially, add 10 mL of tetraethylene glycol, 10 mmol (3.29 mL) of oleylamine, and 8 mmol (2.55 mL) of oleic acid, using a pipette.
   Note: After removing oleyamine and oleic acid bottles from the refrigerator, place them into a water bath at 35°C until completely melted, then take the required volume using a pipette.
3. Prepare two 13.5-mL glass vials.
4. Weigh 0.2 mmol of cobalt (II) acetylacetonate, and 0.2 mmol of iron (III) acetylacetonate. Transfer them to a glass vial labeled as Co and Fe precursors. Then, sequentially, add 2 mL of oleylamine, and 1 mL of toluene.
5. Weigh 0.1 mmol of silver nitrate, and transfer it to the remaining glass vial labeled as Ag precursor. Then, sequentially, add 1 ml of oleylamine, and 1 mL of toluene.
6. Seal the caps of the two vials with a parafilm membrane, and place them in a sonicator with High Power Mode, for complete dissolution of all the reagents.
   Note: To dissolve the reagents quickly, the vials could be warmed at approximately 40°C during this process. There is no time limit for this step, but they should be ready before the injection steps.
7. Prepare two septums, as shown in Figure 2.
   Note: Use the 12G-needle to make a hole, to insert the Pasteur pipette into the rubber septum. Adding some ethanol to the hole makes the insertion easier. Ethanol will evaporate after insertion.

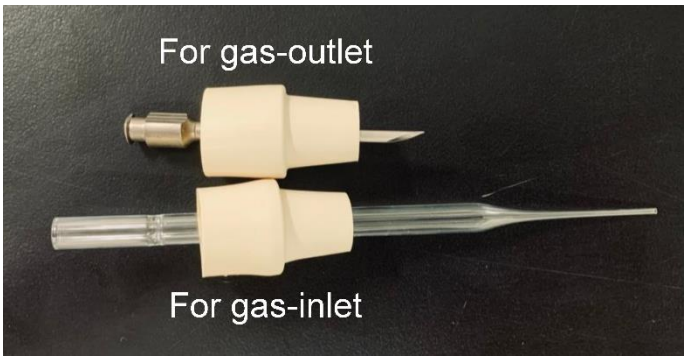


Figure 2. The septum rubbers prepared for Ar bubbling.
Later on, the needle is removed from the septum (top) to insert a thermocouple.

8. Place the three-neck round bottom flask containing the raw reaction materials (prepared in Step A2) on the mantle heater.
9. Connect the trap sphere to the center neck of the flask, and hold them with a clamp. Then, plug in the condenser tube, and connect the other end of the condenser to a trapper containing liquid paraffin (Figure 3).
Note: The reflux condenser uses air without running water.

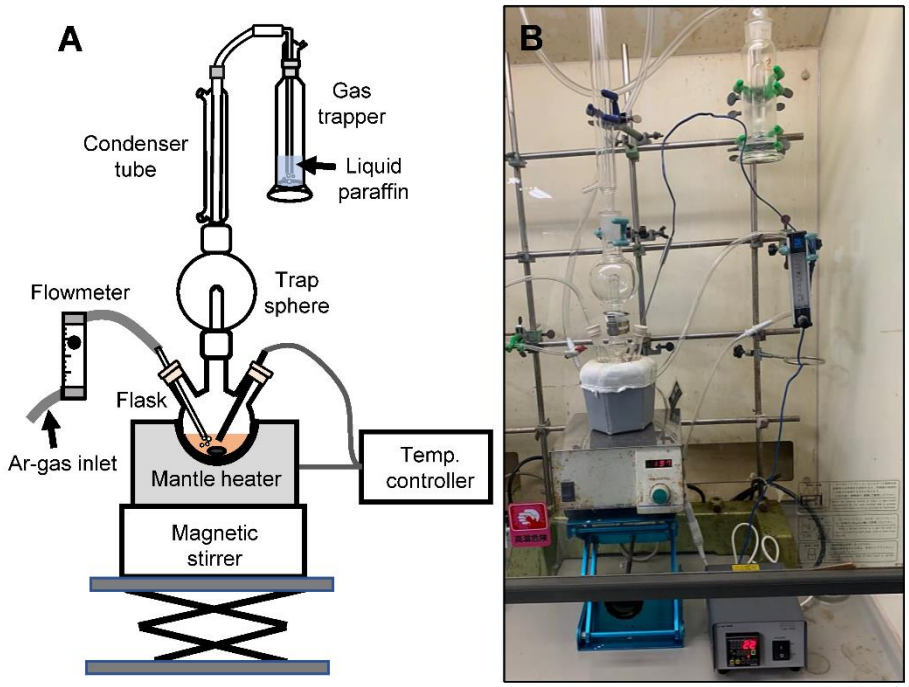


Figure 3. The illustration (A) and photograph (B) of the experimental setup for preparing MPNPs.

10. Seal the two remaining open necks using the septums shown in Figure 2.
Note: The tip of the Pasteur pipette for Ar bubbling should be dipped into reaction solution, but not touch the magnetic stir bar.
11. Turn on the magnetic stirrer at a speed of ~150 rpm, and pump in Ar gas through the Pasteur pipette, at a flow rate of 0.35 L/min. The gas flows out through the 12G-needle. Leave it for 5 min, to complete the replacement of the atmosphere in the flask with Ar gas.

12. Remove the needle, and insert a thermocouple.
13. Turn on the temperature controller, and set the temperature to 100°C.
    Note: Due to the high viscosity of the solution at RT, the stirring speed cannot be set immediately at 500 rpm. Therefore, while the temperature increases, increase the stirring speed slowly. At 50°C, the stirring speed could be fully set at 500 rpm. The heating rate of this step is about 12−13°C/min.
14. From the moment the temperature reaches 100°C, maintain it for 10 min. During this step, the silver seeds are formed.
    Note: Overheating may be observed, in which the temperature is elevated above 100°C. Slightly lowering the heating mantel to reduce its contact with the flask will reduce the temperature.
15. After 10 min, increase the temperature to 250°C, by setting the temperature controller at 300°C. *Note: The purpose of this setting is to linearly rise the temperature up to 250°C (Figure 4).*

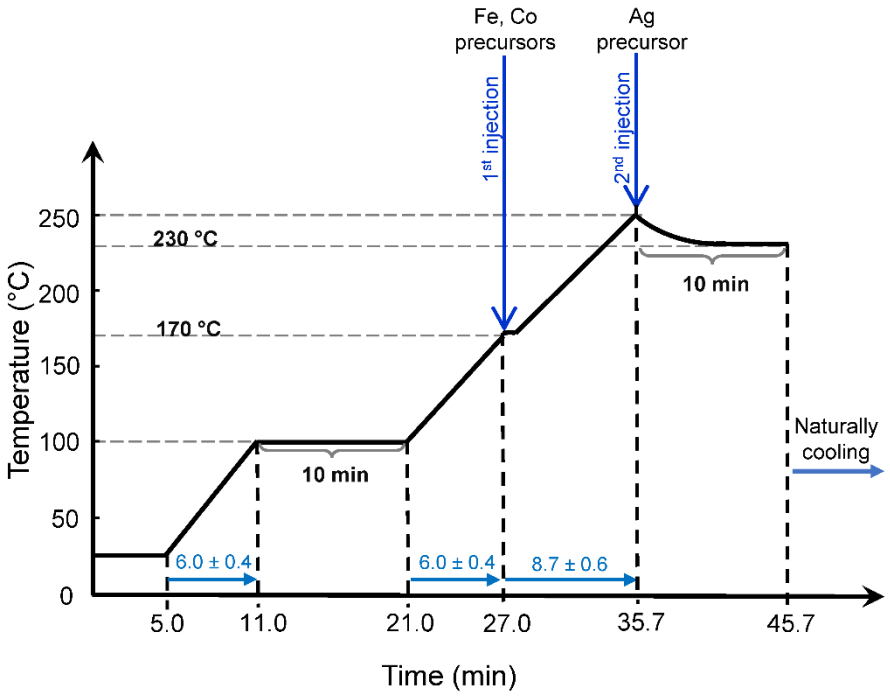


Figure 4. Temperature profile of the preparation process of MPNPs.

16. During the temperature increase, once it reaches 170°C, inject the pre-prepared mixture of Fe and Co precursors, using a glass syringe and 20 G-needle.
    Note: The needle is inserted via the septum containing the thermocouple. Inject the solution drop by drop at a fast pace, for a duration of 1 min. The temperature should be kept around 170°C, by slightly lowering the heating mantle to reduce contact with the flask. The heating rate from 100°C to 170°C is about 12°C/min.
17. Once the temperature reaches 250°C, inject the pre-prepared Ag precursor using a glass syringe and another 20G-needle. Then, immediately reset the temperature of the controller to 230°C. Maintain the reaction for 10 min.
    Note: This is the most important step. The injection of Ag precursor is normally done in about 20 s. It should not be less than 10 s, or longer than 30 s. After the injection, slightly lower the heating mantle to reduce contact with the flask to avoid overheating. We confirmed that reaction time can be prolonged to 15 min, but the quality of the MPNPs was not influenced. The heating rate from 170°C to 250°C is about 9°C/min.
18. Set the temperature controller to 0°C to stop heating, and remove the mantle heater. Wait for the reaction

system to cool off naturally, while continuing stirring and Ar bubbling. At this time, some of the synthesized particles will be attached to the magnetic stirring bar, but they will be redispersed again during the cooling process.

19. Once the temperature of the reaction solution is less than 70°C, stop Ar gas flow, and turn off the magnetic stirrer.
20. Carefully disassemble the setup, and use a pipette to transfer the reaction solution from the three-neck flask to two 50-mL centrifuge tubes evenly.
21. Add acetone, to fill the tube to 45 mL. Then, perform centrifugation using a Kubota 5910 at 4,640 × *g* and RT for 5 min.
22. Discard all supernatant, and add 400 µL of hexane to each tube, for redispersion of MPNPs.
23. Use a micropipette to transfer 200 µL of redispersed MPNP solution to two other 50-mL tubes. Subsequently, fill with acetone up to 45 mL in total, and perform centrifugation using Kubota 5910 at 4,640 × *g* and RT for 5 min.
24. Discard the supernatant, and redisperse the obtained MPNPs in 3 mL of chloroform. Determine the concentration of MPNP dispersion through the absorption spectrum, using an ultraviolet-visible absorption spectrophotometer.
    Note: The concentration of MPNPs was determined using a calibration curve of y = 0.024x, where y was the absorption peak value of localized surface plasmon resonance of MPNPs, and x was the concentration of MPNPs (µg/mL).
25. Store obtained MPNPs in chloroform at 4°C, in a glass vial with closed-top cap. Seal it with Parafilm.

B. Encapsulation of MPNPs in PEGylated phospholipids

1. Prepare a 50-mL two-neck round bottom flask (Figure 5A).
2. Pour 3 mL of MPNPs dispersed in chloroform at a concentration of 1 mg/mL into the flask. Subsequently, add 1,350 µL of 18:1 glutaryl PE (5.5 mM) in chloroform, and 900 µL of PEG350-DOPE (5.5 mM) in chloroform to the dispersion.
3. Seal using septum rubbers, place the septum containing the Pasteur pipette in the center neck, and the septum containing the needle in the angled neck.
   Note: The tip of the Pasteur pipette should not dip in the dispersion.
4. Pump in Ar gas at a rate of 0.5 L/min, to completely evaporate the chloroform (Figure 5B).
   Note: In this step, the inert gas could be either Ar or N_2.

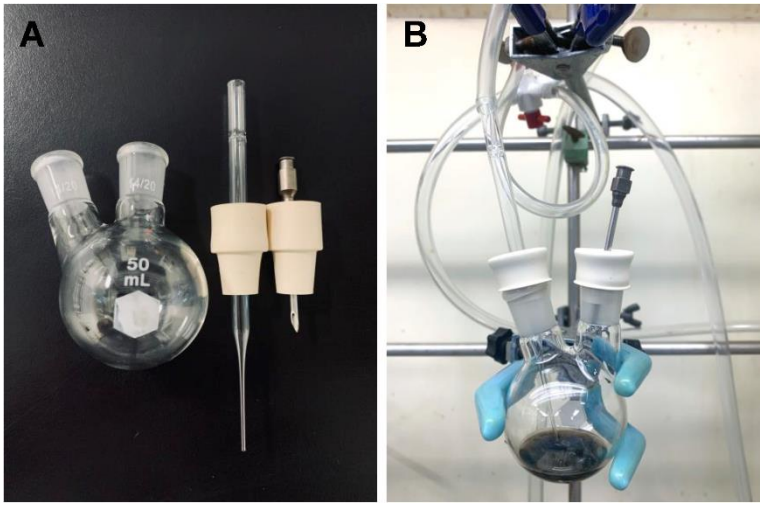


Figure 5. The glassware (A) and the experimental setup (B) for encapsulation of MPNPs in PEGylated phospholipids.

5. Add 1.5 mL of deionized water to redisperse the obtained precipitation. Collect the dispersion into two 1.5-mL centrifuge tubes.
6. Centrifuge at 1,400 × *g* and RT for 5 min, using the Hitachi CF15RXII high-speed micro centrifuge, to eliminate big aggregated particles.
7. Carefully transfer the supernatant from the centrifugated tubes into two new 1.5-mL tubes. Then, centrifuge at 86,600 × *g* and 4°C for 10 min, using the CS100FNX ultracentrifuge, to remove empty micelles.
   Note: In this step, if the MPNPs were not completely collected, increase the centrifugation speed up to 100,000 × g.
8. Discard the supernatant, and redisperse the obtained particles in 1 mL of deionized water.
9. Determine the concentration of phospholipid encapsulated MPNPs from the absorption spectrum.

C. Conjugation of aDxt using EDC coupling reaction

1. Add 20 mL of MES buffer (pH ~6) to a 100-mL one-neck round bottom flask, containing a magnetic stirrer.
2. Set the magnetic stirrer to 600 rpm.
3. Add 1 mL of phospholipid encapsulated MPNPs dispersion (1 mg/mL) into the flask. Then, sequentially add 125 µL of EDC (200 mM) in deionized water, and 250 µL of NHS (200 mM) in MES buffer. Leave it at RT for 30 min.
   Note: After taking them out from the refrigerator, equilibrate the EDC and NHS to RT before use.
4. Transfer the obtained reaction mixture into 20 × 1.5-mL tubes.
5. Centrifuge at 86,600 × *g* and 4°C for 10 min, using the CS100FNX, and carefully remove the supernatant.
6. Use a micropipette to collect, and redisperse the obtained particles in 1 mL of PBS.
7. Prepare a 100-mL one-neck round-bottom flask, containing 19 mL of PBS.
8. Add 50 mg of aDxt into the PBS solution in the flask (prepared in Step C7), and wait for it to completely dissolve under magnetic stirring.
9. Add the PBS dispersion of MPNPs (prepared in Step C6) into the PBS solution of aDxt (prepared in Step C8). Then, maintain the reaction at RT for 90 min.
10. Transfer the obtained mixture to 20 × 1.5-mL tubes. Then, perform centrifugation at 60,000 × *g* and 4°C for 10 min, using the CS100FNX. Collect, and redisperse the obtained particles in 1 mL of PBS.
11. Determine the concentration of aDxt-conjugated MPNPs (aDxt-MPNPs) in the dispersion, using the absorption spectrum.

D. Pulse-chase experiments for studying intracellular trafficking of MPNPs

Notes:

a. *In a pulse-chase experiment, the pulse is the incubation of aDxt-MPNPs with COS-1 cells for a certain period (t_{load}). After the loading step, the excess amount of aDxt-MPNPs that are not incorporated in the cells is removed. Cells are further incubated in a fresh culture medium for a given period, t_{chase}. The purpose of this experiment is to find the optimal t_{chase}, for aDxt-MPNPs to reach lysosomal compartments through the endolysosomal pathway.*
b. *These experiments are performed on a clean bench under sterile conditions.*

1. Place 10–20 sterilized round cover glasses into a 10-cm culture dish.
2. Add 5 mL of 0.01% PLL solution into the 10-cm dish, and dip the round cover glasses in PLL solution at RT for 5 min, using a tweezer.
3. Remove the PLL solution, cover with aluminum foil, with the foil partially opened, and naturally dry overnight on a clean bench.
4. The next day, wash the PLL coated cover glasses three times, using 5 mL of PBS buffer.
5. Place the four cover glasses in each well of a 24-well plate using a tweezer, which correspond to different t_{chase} values of 1 h, 2 h, 4 h, and 7 h.
   Note: The number of cover glasses increases according to the number of investigated t_{chase}. In the case of

COS-1 cells, the maximum t_{chase} was performed at 7 h. However, it should be noted that the length of t_{chase} depends on different cell lines. Therefore, the incubation time of this experiment could be customized easily.

6. Seed 20,000 COS-1 cells/well and incubate overnight in DMEM (+10% FBS) at 37°C under 5% CO_2.
7. Check the health and confluency of cells, under a bright-field microscope (Keyence, model: BZ-X810) in advance.
8. The next day, remove the culture medium, and wash cells with 500 µL of PBS at RT.
9. For cell starvation, add 0.5 mL of pre-warmed DMEM without FBS, and incubate for 30 min at 37°C under 5% CO_2.
10. Approximately 10 min before finishing the starvation process, add MPNPs to DMEM (+10% FBS), to prepare a dispersion of MPNPs with the concentration of 100 µg/mL.
11. Immediately affter starvation, replace DMEM without FBS with 500 µL of MPNPs dispersion in DMEM (+10% FBS) (prepared in Step D10), and incubate for t_{load} = 1 h at 37°C under 5% CO_2.
12. After 1 h incubation, remove the dispersion, and wash with 500 µL of PBS once. Then, add 500 µL of pre-warmed DMEM (+10% FBS), and incubate for t_{chase}: 0 h, 2 h, 4 h, and 7 h at 37°C under 5% CO_2.
    Note: The t_{chase} would be varied in different cell lines. Therefore, the incubation period could be customized appropriately.
13. After completing the t_{chase}, wash with 500 µL of PBS three times, and add 500 µL of 4% PFA at RT to each well for 15 min.
    Note: Since PFA is a toxic chemical, personnel must wear a lab coat and chemically protective gloves. This step should be performed in a clean bench equipped with a ventilation system and a protective sash. Additionally, keep PFA solution away from flame or heat sources. The PFA should be properly disposed of as hazardous waste. After fixation, the following steps could be performed outside the clean bench. The sterile conditions are not required.
14. Wash with 500 µL of PBS three times, add 500 µL of 50 µg/mL digitonin-PBS for permeabilization to each well, and wait for 5 min.
15. Wash with 500 µL of PBS three times, add 500 µL of 50 mM NH_4Cl-PBS to each well, and wait for 10 min.
16. Wash with 500 µL of PBS three times, and perform blocking by adding 500 µL of 3 wt% BSA-PBS to each well, and waiting for 30 min.
17. For each well, add 500 µL of 3 wt% BSA-PBS containing 2 µL of AF647@CD107A for staining the lysosomes, and 0.25 µL of 100 µg/mL DAPI for staining the nuclei. Wait at RT for 1 h, or keep it at 4°C overnight.
18. Wash with 500 µL of PBS three times. For each washing step, wait for 5 min after adding PBS.
19. Add a drop of antifade mounting medium onto a white slide glass edge grinding, carefully take the cover glass using tweezers, and place it onto the glass substrate for observation with the cell-facing surface in contact with the mounting medium. Ensure there are no bubbles and remove extra fluid if necessary.
20. Leave it in a dark place for several hours until it is completely dry. Then, observe the samples using a CLSM.

E. Observation of MPNPs-loaded cells under CLSM

1. Select 405, 473, and 635 nm lasers for the excitation of DAPI, aDxt-MPNPs, and AF647, respectively.
2. For DAPI dye, select the barrier filter (BA) 435–455 nm.
3. For plasmonic scattering signal of aDxt-MPNPs, select no barrier filter, as, unlike the fluorescent dye, the scattering signal from aDxt-MPNPs has the same wavelength as the laser wavelength.
4. For AF647 dye, select BA 655–755 nm.
5. Capture CLSM images of more than five different randomly-selected regions. Record DAPI signal separately to plasmonic scattering signal and AF647 fluorescence (Figure 6).
6. Perform colocalization analysis of aDxt-MPNPs and lysosomes, by determining the threshold Manders' colocalization coefficient (R_t), using ImageJ software.

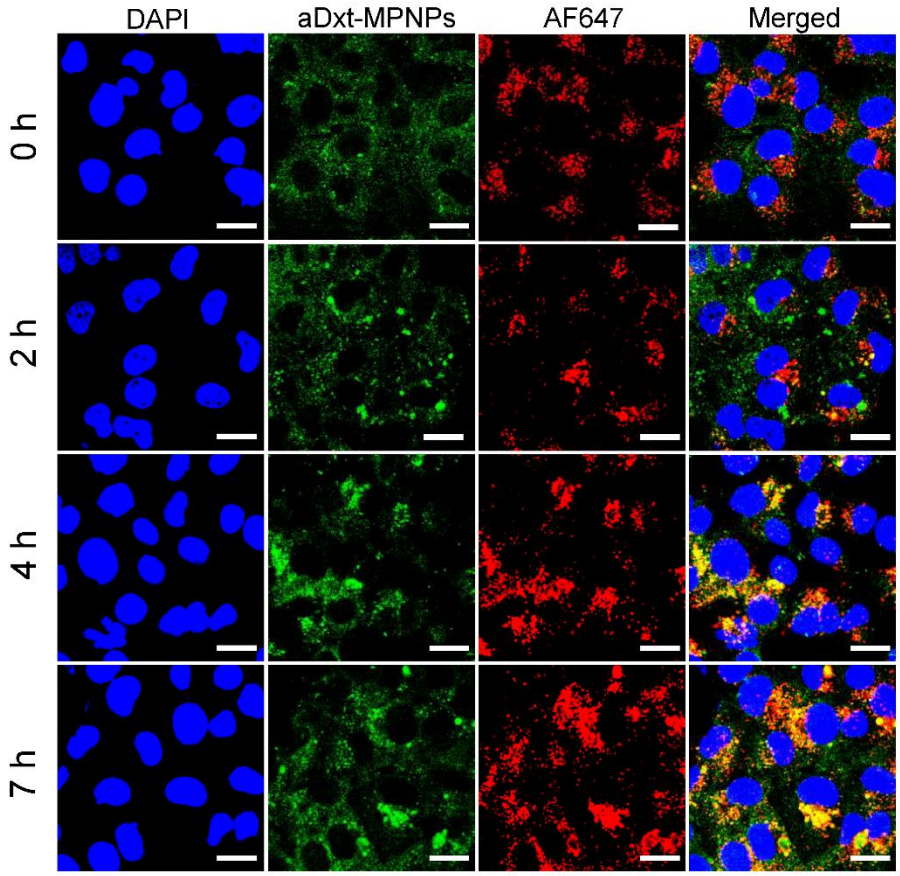


Figure 6. CLSM images of aDxt-MPNP-loaded COS-1 cells at different t_{chase} values of 0 h, 2 h, 4 h, and 7 h, captured by CLSM (scale bar: 20 μm).
Nuclei (blue) and lysosomes (red) are stained by DAPI and AF647, respectively, as described in the text. aDxt-MPNPs were observed by plasmonic scattering signals. The merged images were obtained using ImageJ. Adapted with permission from Le *et al.* (2022). Copyright 2022 American Chemical Society.

F. Accumulation of aDxt-MPNPs to lysosomes, homogenization, and magnetic isolation of lysosomes

1. Seed 2 × 10^6 COS-1 cells/dish for two 10-cm dishes, and incubate in DMEM (+10% FBS) for 24 h.
2. Check the health and confluency of cells under a bright-field microscope (Keyence, model: BZ-X810) in advance.
   Note: The health of COS-1 cells was checked by confirming their adherent status on a cell dish, using bright-field microscopy. In addition, the possibility of contamination was also checked at the same time. The confluency of cells was estimated to be less than 80% in this particular experiment. However, it would change depending on the cell type. The desired confluency of cells would be ranging from 70% to 80%. If cells are well adhered on the dish without being contaminated, and with around 70-80% confluency, one can go to the next step.
3. For cell starvation, add 5 mL of pre-warmed DMEM without FBS to each cell dishes, and incubate at 37°C under 5% CO_2 for 30 min.
4. About 10 min before finishing the starvation process, add MPNPs to DMEM (+10% FBS), to prepare a 10-mL of dispersion of MPNPs, with a concentration of 100 μg/mL.
5. Immediately after starvation, replace DMEM without FBS with 5 mL of MPNPs dispersion in DMEM (+10% FBS) (prepared in Step F4) to each cell dish, and incubate at 37ºC under 5% CO_2 for 8 h.
   Note: In this study, we chose the t_{load} = 8 h for loading. t_{load} strongly affects the isolation yield of lysosomes.

This parameter could be prolonged depending on the cytotoxicity of MPNPs to the cells.

6. Discard the aDxt-MPNPs containing medium, and wash with pre-warmed PBS once.
7. Add 5 mL of DMEM (+10% FBS), and incubate further for t_{chase} = 7 h. The optimal t_{chase} has been already determined in the pulse-chase experiment section. Depending on the cell type, this parameter may vary.
8. Place necessary equipment, including the magnetic column, MidiMACS separator, 2.5-mL syringe with 23G-needle (Terumo syringe with needle 2.5 mL 23G blue), and 5-mL tubes, into the cold room, where the temperature is maintained at 4°C, for at least 30 minutes before the pulse-chase experiment, to equilibrate the temperature. If a cold room is not available, use an ice-box to store the equipment instead.
9. Discard the medium, and wash the cells with PBS.
10. Add 1.5 mL of cold PBS to each culture dish, and place them on ice.
11. Scrape off the cells using a Cell Lifter, and transfer them from both culture dishes to a 15-mL centrifuge tube. Centrifuge at 190 × *g* and 4°C for 4 min, using the Kubota 5910 with a ST-720 swinging bucker rotor.
    Note: In this step, the amount of particle uptake could be qualitatively evaluated via the color of the cell pellet (Figure 7). The darker the color, the higher number of particles internalized. If the cell color is still white, it means a very low uptake efficiency. The isolation of lysosomes may fail.
12. Discard the supernatant, and add 1 mL of ice-cold PIS to re-suspend the cell pellet. Then, transfer to a 5-mL tube, and keep in an icebox.
    Note: After this step, the experiments are continued in a low-temperature room, where the temperature is maintained at 4°C.

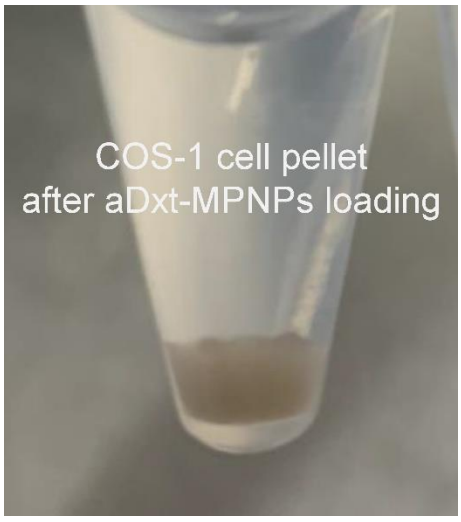


Figure 7. A photograph of aDxt-MPNPs loaded COS-1 cells with t_{load} = 8 h and t_{chase} = 7 h.

13. Use a 2.5-mL syringe with a 23G-needle, and repeatedly (15 passages) pass the cell suspension through the syringe, to homogenize the cells.
    Note: The optimal number of passages must be determined experimentally (Figure 8). The low homogenization efficiency could obviously affect the yield of lysosome isolation. In contrast, homogenization efficiency enhanced by increasing the number of passages may also lead to lysosomes being broken. Therefore, in this study, a small portion of unbroken cells or large cell fragments is left over.

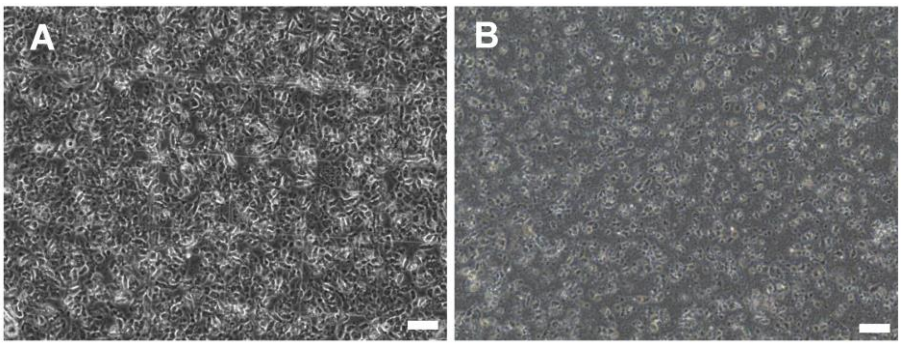


Figure 8. The bright-field image of COS-1 cells under phase contrast mode.
Before (A) and after (B) homogenization using a syringe with a 23G-needle (15 passages). Before homogenization, cells can clearly be seen as a high density of dark areas encircled by bright halos. After homogenization, the number of cells is reduced, and the cell mixture becomes a slurry, due to the breaking of the cell membranes. Consequently, the number of bright halos decreases significantly. A small portion of either unbroken cells or large cell fragments in the slurry can still be observed. Scale bar, 100 µm.

14. Place an MS Column in a MidiMACS separator.
    Note: Another type of MACS® Column, such as LS Column, could also be used in this experiment.
15. Equilibrate the MS Column, by adding 1 mL of PIS. Discard the flow-through.
16. Transfer the cell lysate (prepared in Step F13) to the MS Column, using the micropipette, and allow the cell lysate to pass through the column. The magnetic fraction will be trapped inside the column, while the nonmagnetic fraction will pass through the column.
17. Discard the flow through. Wash the column using 1 mL of PIS twice, to further eliminate unbound materials.
18. Remove the MS Column from the MidiMACS separator.
19. Add 0.5 mL of PIS, and insert the plunger to collect the magnetic fraction containing lysosomes in a 1.5-mL microtube. Repeat this step once again.
20. Centrifuge the obtained suspension at 5,000 × *g* and 4°C for 10 min, using an MDX-310 system.
    Note: This step is to remove the remaining soluble proteins in the isolated fraction.
21. Discard the supernatant, and redisperse the obtained lysosome pellet in 100 µL of PIS.
    Note: If the isolated lysosome fraction is subjected to proteome analysis, re-suspend the pellet in 50 mM triethylammonium bicarbonate.

Data analysis

The colocalization analysis of aDxt-MPNPs and lysosomes is performed using ImageJ. First, open the image, then choose "Plugin" → "Segmentation" → "Color Clustering". Afterward, in the new window, in the "Channel" section, choose the appropriate color for the image. For this specific case, the illustrated colors of aDxt-MPNPs and lysosomes are green and red, respectively. Next, press "Run", and then choose "Show result" to obtain the segmented image. Note that only one image of either aDxt-MPNPs or lysosomes can be processed at once (Figure 9). After obtaining segmented images, the R_t represents the percentage of lysosomes overlapped with aDxt-MPNPs. To open color clustered images of aDxt-MPNPs and lysosomes, select "Analyze" → "Colocalization" → "Coloc 2". In the new window, choose the image of aDxt-MPNPs for channel 1, and the image of lysosomes for channel 2, then check the Manders' correlation box, and press OK (Figure 10). Repeat this step for five pairs of images from each experimental condition.

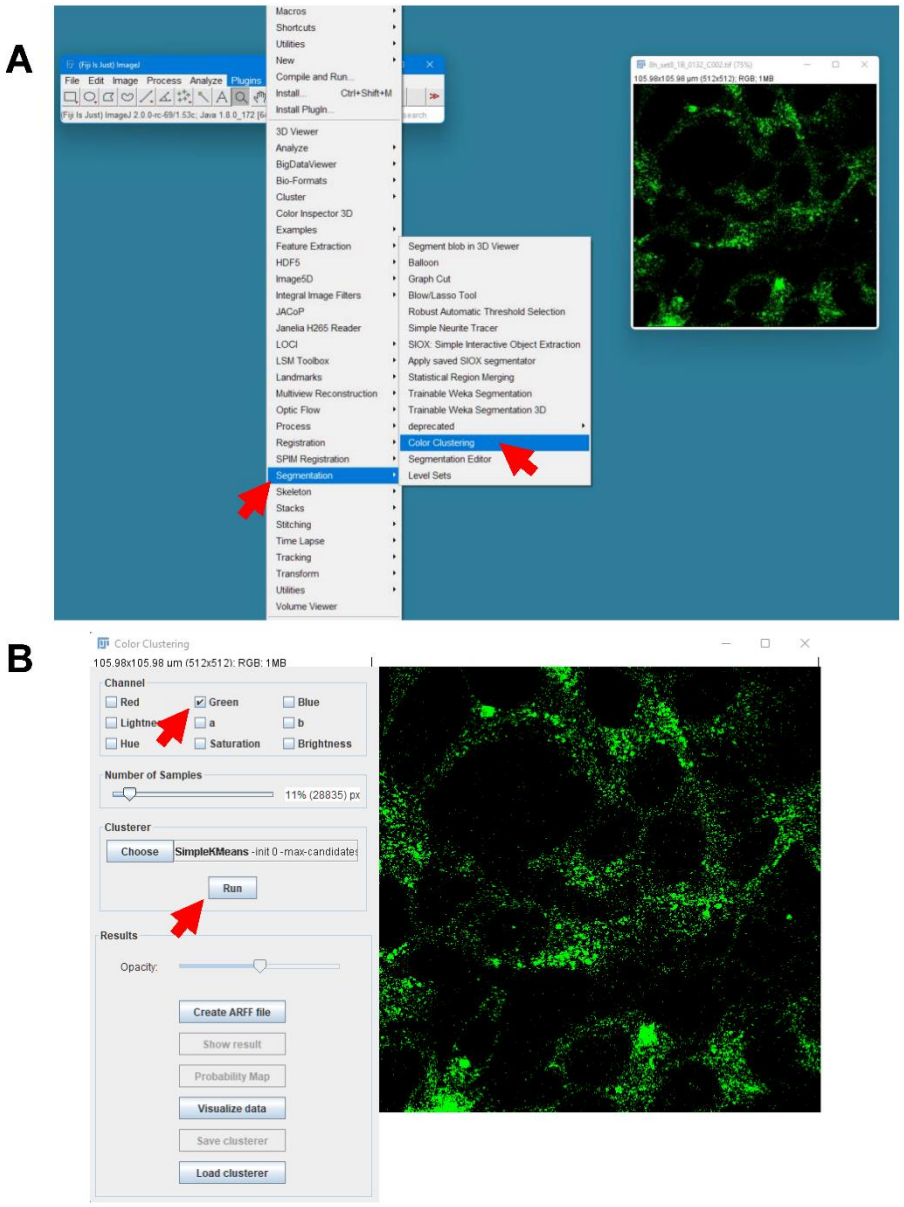


Figure 9. Segmentation of CLSM images using color clustering.
(A) Select "Plugin" > "Segmentation" > "Color Clustering". (B) For an aDxt-MPNP image, the illustrated color of nanoparticles is green, therefore, select green (in the channel section), and click "Run". Repeat this step for the CLSM image of stained lysosomes, and select the appropriate color for segmentation accordingly.

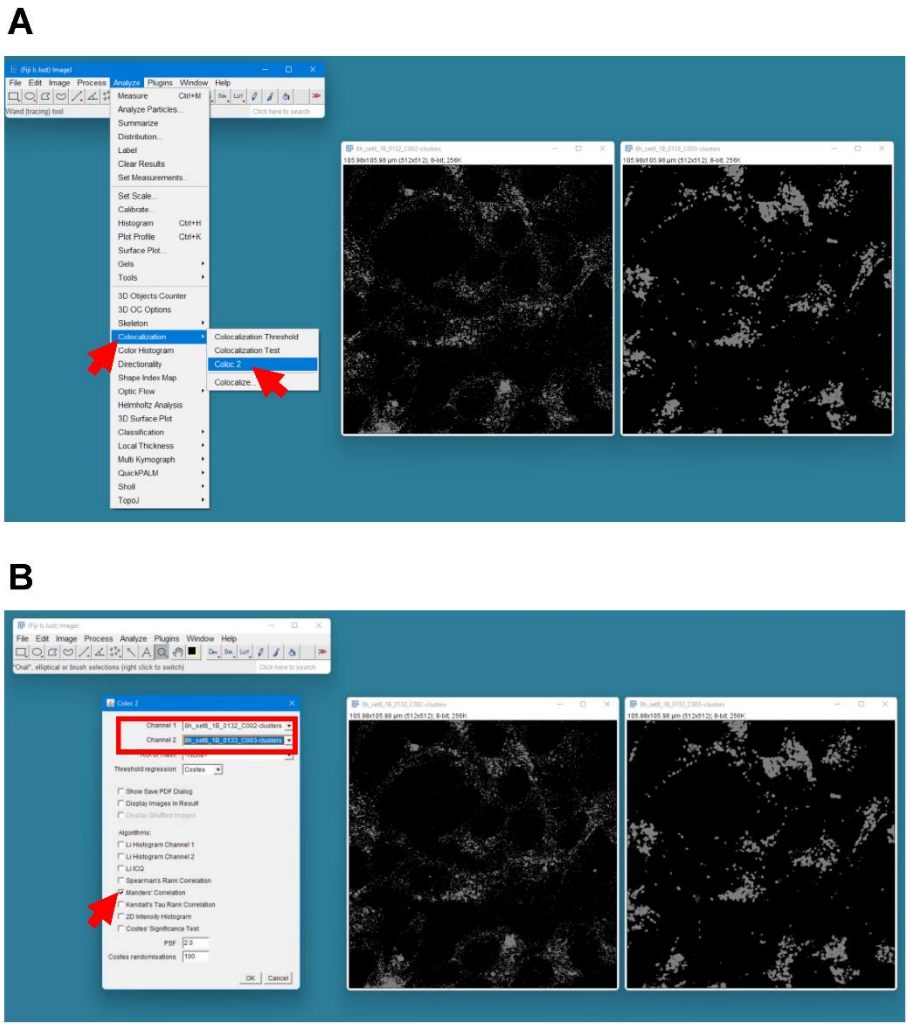


Figure 10. The process to determine the R_t using Coloc 2 plugin in ImageJ software.
(A) Open two segmented images in the previous steps, select "Analyze" > "Colocalization" > "Coloc 2". (B) In the new window, select images for channel 1 and channel 2. Then, check the Manders' correlation box, and click OK.

After completing the image analysis, a graph of R_t-versus-incubation time can be constructed (Figure 11A). The incubation time is the sum of t_{load} and t_{chase}. As aDxt-MPNPs are transported to lysosomes, the R_t value increases. However, due to the limited spatial resolution of the CLSM image, the R_t value is saturated. From this graph, the value t_{chase} can be determined. Normally, the t_{chase} is chosen after one time when R_t reaches a plateau. The reasoning behind this is that, in the endolysosomal pathway, late-endosomes are fused with lysosomes, which could also result in the high colocalization of aDxt-MPNPs with lysosomes. However, it is recommended that, after obtaining the isolated lysosome fraction, the level of late endosomes should be evaluated using Western blot analysis (Figure 11B). If the late endosome still exists, a further prolonged t_{chase} is necessary.

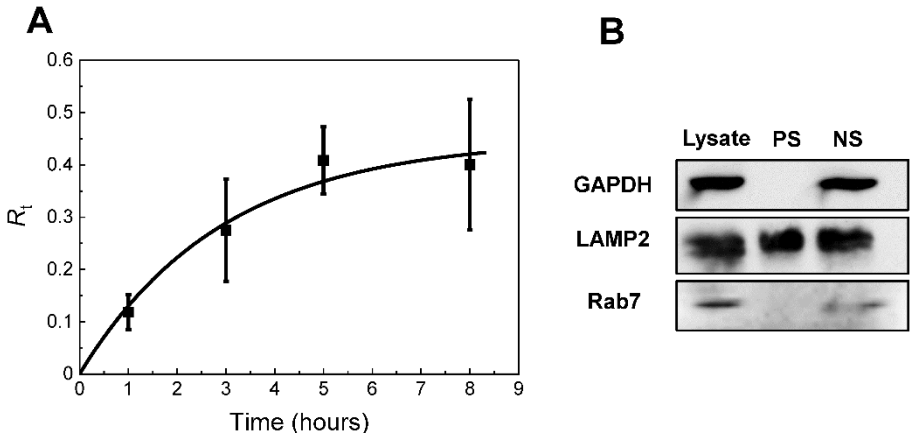


Figure 11. Time-lapse colocalization of aDxt-MPNPs with lysosomes and Western blot of cell lysate, PS, and NS fractions.
(A) The graph of R_t-versus-incubation time. The accumulation of aDxt-MPNPs in lysosomes is indicated by the increase of R_t over time. (B) A western blot of the isolated lysosome fraction. PS: positive selection (magnetic fraction); NS: negative selection (nonmagnetic fraction); GAPDH: glyceraldehyde-3-phosphate dehydrogenase (cytosolic protein as a control); LAMP2: lysosomal associated membrane protein 2 (lysosome marker), Rab7: late endosome marker protein. Adapted with permission from Le *et al.* (2022). Copyright 2022 American Chemical Society.

Recipes

1. MES buffer (0.1 M, pH ~6)
Dissolve 3.90 g MES in 180 mL of deionized water. Monitor the pH of the solution using a pH meter, then take 10 N sodium hydroxide aqueous solution using a micropipette, to adjust the pH of the solution to approximately 6. Then, add water up to 200 mL. Sterilize the solution by filtration through a 0.2-μm filter before use. Store in a dark colored bottle.

2. PBS buffer
Dissolve 9.6 g of PBS in 1 L of deionized water. The solution should be sterilized by an autoclave before use. Store the solution at 4°C.

3. Digitonin solution
Dissolve 25 mg of digitonin in 500 μL of dimethyl sulfoxide (DMSO). Divide the solution into microtubes, at 15 μL/tube. Store the solution at -20°C. It is diluted with PBS, for permeabilization.

4. Ammonium chloride solution
Dissolve 0.160 g NH_4Cl in 60 mL of PBS buffer. Store the solution at 4°C.

5. DAPI staining solution
From the commercial product, prepare the DAPI stock solution with a concentration of 100 μg/mL, store in the refrigerator at 4°C. For nucleus staining, dilute the stock solution 2000 times further.

6. Protease inhibitor solution (PIS)
a. Prepare 20 mL of PBS in a 50-mL tube.

b. Add 20 µL of 0.1 M phenylmethylsulfonyl fluoride and 100 µL of protease inhibitor cocktail to the tube. The dilution factor is about 1000× and 200× for phenylmethylsulfonyl fluoride and protease inhibitor cocktail, respectively.
c. Disperse the solution homogeneously using a vortex. Then, keep the solution in an ice box.

Note: This solution should be prepared at the time of use. Long-term storage is not recommended. The composition of the inhibitor cocktail is: 0.1 mg/mL leupeptin hemisulfate monohydrate; 0.14 mg/mL pepstatin A; 14 mg/mL N-p-tosyl-L-phenylalanine chloromethyl ketone; 15 mg/mL N_{α}-p-tosyl-L-arginine methyl ester hydrochloride; 0.4 mg/mL aprotinin; 32 mg/mL benzamidine dissolved in DMSO. The inhibitor cocktail can be prepared in advance and stored in small tubes at -20°C.

Acknowledgments

This protocol is derived from the original research paper, Le *et al.* "Quick and Mild Isolation of Intact Lysosomes Using Magnetic−Plasmonic Hybrid Nanoparticles" ACS Nano 2022 Jan 3; 16(1): 885–896. doi: 10.1021/acsnano.1c08474 (Le *et al.*, 2022). This work was partly funded by the Grant-in-Aid for Young Scientists from the Japan Society for the Promotion of Science (grant no. 21K14506) to M.T.

Competing interests

The authors declare no competing interests.

Ethics

No human or vertebrate animal subjects are used in this study.

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