Chapter 37

Super-Resolution Imaging of Intracellular Lipid Nanocarriers to Study Drug Delivery in Photodynamic Therapy

Enzo M. Scutigliani, Jakub A. Kochan, Emilie C. B. Desclos, Art Jonker, Michal Heger, and Przemek M. Krawczyk

Abstract

Liposomal nanocarriers are intensively investigated as delivery vehicles for photoactivatable agents used in photodynamic therapy (PDT). The uptake, intracellular distribution, and processing of the nanocarriers are of paramount importance for the effectiveness of the therapy; visualization and analysis of these processes can, therefore, stimulate the development of improved PDT modalities. Here we describe a simple protocol, based on super-resolution imaging, that can be used for detailed quantification of concentration, distribution, and size of individual lipid nanocarriers in adherent mammalian cells.

Key words Photodynamic therapy, Photosensitizers, Super-resolution microscopy

1 Introduction

During the last decade, photodynamic therapy (PDT) has received considerable amount of interest as a selective anticancer approach. However, several drawbacks prevent PDT from being routinely applied. Most of these drawbacks relate to the poor intra-tumoral delivery of photosensitizer molecules [1]. To overcome this problem, intensive efforts are directed towards the development of photosensitizer nanocarriers serving as drug delivery agents [1, 2]. The incorporation of photosensitizers into liposomes has been shown to be a promising approach, but a deeper understanding of how these carriers perform their role is required for optimal utilization in PDT.

All liposomes share the common trait that their cellular uptake depends on their interaction with membranes of target cells. Therefore, the ability to visualize and study the behavior of nanocarrier-originating lipids in tumor cells can provide a deeper understanding of nanocarrier performance. By incorporation of a compatible

Mans Broekgaarden et al. (eds.), *Photodynamic Therapy: Methods and Protocols*, Methods in Molecular Biology, vol. 2451, https://doi.org/10.1007/978-1-0716-2099-1_37,

fluorophore in the liposome of interest, the behavior of the nanocarrier with respect to lipid dynamics can be observed with the use of various microscopic imaging techniques, including super-resolution by ground-state depletion followed by individual molecule return (GSDIM) microscopy [3, 4]. Earlier studies have indeed demonstrated the benefits of visualizing lipid nanocarriers [5, 6], including fluorescence microscopy and super-resolution microscopy techniques [7–10].

Here, we provide a simple protocol for visualizing and analyzing the subcellular localization and morphology of nanocarrier-originating lipids at nanometer resolution with the use of GSDIM super-resolution microscopy. GSDIM is mostly realized on cells attached to glass coverslips. Other setups are possible, such as imaging tissue samples, but require adapted protocols. For these reasons, only the protocols for cells plated on coverslips are described here. This method, based on a previously reported technique and protocol [11], can yield information on membrane-nanocarrier interactions, supporting the development of lipid nanocarriers for PDT.

2 Materials

Store all materials at room temperature unless stated otherwise.

2.1 UltraClean Coverslips

1. Coverslips, 24-mm diameter, thickness #1.5 (0.16–0.19 mm).
2. 2 M HCl.
3. 0.1 M $Na_2B_4O_7$, pH = 8.5.
4. 2 M NaOH.
5. 20% H_3PO_4; **Warning**: This reagent is highly toxic, so use appropriate protection.
6. 70% High-grade ethanol.

2.2 Fluorescently Labeled Liposomes

The versatility of this protocol was validated by using different formulations of endothelium-targeting liposomes (ETLs), in which phosphatidylcholine (PC) conjugated to the fluorophore nitrobenzoxadiazole (NBD) was added at the expense of dipalmitoylphosphatidylcholine (DPPC) as described elsewhere [12, 13].

2.3 Paraformaldehyde Fixation

1. Phosphate-buffered saline (PBS), pH = 7.4, $CaCl_2$/$MgCl_2$-free.
2. 4% Paraformaldehyde (PFA), methanol-free.
3. 0.5% Triton X-100 in PBS.
4. 5% Bovine serum albumin (BSA) in PBS.

2.4 Immunolabeling

1. 5% BSA.
2. Primary and secondary antibodies, prepared as specified by the manufacturer.
3. 0.1% Tween-20.
4. Parafilm.

2.5 OxeA Imaging Buffer

1. Sodium DL-lactate, 60% solution: Store 100 μL aliquots at 4 °C.
2. 0.5 M Monoethanolamine (MEA), pH = 8: Store 110 μL aliquots at −20 °C.
3. 5 M NaOH.
4. OxyFluor (Sigma-Aldrich): Store 40 μL aliquots at −20 °C.
5. PBS.

2.6 Sample Mounting

1. Chamlide CM-B25-1 magnetic chamber (Live Cell Instrument, Seoul, Korea).
2. Heavy mineral oil.

3 Methods

Carry out all procedures at room temperature unless specified otherwise. Prepare and store all reagents at room temperature unless indicated otherwise. Prepare all solutions using ultrapure water. The fixation, labeling, and imaging solutions should always be freshly prepared from stock solutions.

3.1 Preparation of Ultraclean Coverslips

1. *See* **Note 1** for the rationale of preparing ultraclean coverslips.
2. Place coverslips in a clean glass petri dish.
3. Immerse in 2 M HCl overnight.
4. Wash with ddH_2O.
5. Immerse in 0.1 M $Na_2B_4O_7$ (pH = 8.5) for at least 2 h.
6. Wash with ddH_2O.
7. Immerse in 2 M NaOH for at least 2 h.
8. Wash with ddH_2O.
9. Immerse in 20% H_3PO_4.
10. Wash with ddH_2O.
11. Store in 70% ethanol (high grade).
12. Each of the reagents can be reused for up to 10 procedures.

3.2 Plating Cells and Incubation with Liposomes

1. Place ultraclean coverslips in the wells.
2. Wash with 2 mL PBS per well to remove residual ethanol.
3. Seed a number of cells that are required to reach about 60% confluence on the day of the experiment.
4. Incubate cells for 24 h.
5. Expose cells to liposomes by replacing culture medium with a pre-dilution of liposomes in culture medium.

3.3 Paraformaldehyde Fixation

1. Wash the cells with 2 mL PBS.
2. Fix with 2 mL 4% PFA for 10 min.
3. Wash twice with 2 mL PBS for 5 min.
4. Incubate with 2 mL 0.5% Triton X-100 in PBS for 10 min.
5. Wash twice with 2 mL PBS for 5 min.
6. Incubate for at least 1 hour in 2 mL 5% BSA in PBS.
7. Proceed to the labeling steps.

3.4 Immunostaining (Optional)

1. *See* **Note 2** for general consideration regarding immunostaining in super-resolution light microscopy.
2. Incubate with primary antibody in 5% BSA in PBS.
3. Wash twice with 2 mL 0.1% Tween-20 in PBS for 5 min.
4. Incubate with secondary antibody in 5% BSA in PBS as stated in **step 1**.
5. Wash twice with 2 mL 0.1% Tween-20 in PBS for 5 min.
6. Store the sample in 2 mL PBS. Protect from light.

3.5 Preparation of OxeA Imaging Buffers

1. *See* **Note 3** for the rationale and practical considerations regarding the imaging buffers.
2. Add 50 μL of MEA to a 1.5 mL tube containing 100 μL of sodium DL-lactate.
3. Add 3 μL of NaOH.
4. Add 15 μL of OxeA.
5. Add 350 μL of PBS.
6. Mix well.

3.6 Imaging Setup

1. Mount a coverslip on the magnetic ring holder.
2. Wash the sample with 500 μL of imaging buffer and subsequently cover the sample with 500 μL of imaging buffer to prevent dilution of the buffer.
3. Cover the imaging buffer with 500 μL of heavy mineral oil (*see* **Note 4**).
4. Place the ring on the stage of the microscope.

5. Allow the sample to settle for about 15 min to minimize drift during image acquisition.
6. Proceed to image acquisition as recommended by the microscope manufacturer.
7. *See* **Note 5** for an example image and its interpretation.

4 Notes

1. Coverslips are coated during the manufacturing process to prevent sticking during storage. Removing this layer by washing the coverslips with several reagents reduces background noise and improves image quality.
2. Aspecific labeling appears more pronounced in super-resolution light microscopy compared to other light microscopy methods. We therefore advise to optimize antibody concentration and incubation time for optimal results. In general, we obtained satisfying results by limiting incubation times for the first and secondary antibodies to 45 and 30 min, respectively. To perform the antibody staining, place a 60 μL droplet of antibody solution on a Parafilm layer. Use a bent injection needle to wedge the coverslip off the bottom of the 6-well plate and use forceps to gently place it on the droplet. The cells should be in contact with the labeling solution. Do not press on the coverslip. Cover the coverslip with an opaque lid. After incubation, use the forceps and needle to place the coverslip back into the 6-well plate.
3. GSDIM requires the use of imaging buffers that reduce photobleaching of fluorophores by oxygen scavenging [11]. The OxeA buffer is effective for 2–4 h and can be used for multicolor imaging. However, other imaging buffers can be taken into consideration depending on the sample [11].
4. To extend the oxygen-scavenging capacity of the imaging buffer, the sample is sealed with gas-impermeable oil to prevent the entrance of environmental oxygen in the sample.
5. An example is provided in Fig. 1. Super-resolution imaging allowed us to accurately determine the localization of NBD-conjugated PC originating from ETLs. Incubation of bone osteosarcoma (U2-OS) cells with NBD-ETLs resulted in the accumulation of vesicle-like structures on the cell (Fig. 1a). We visualized and quantified the diameter of these structures to demonstrate the gain in resolution (Fig. 1b, c).

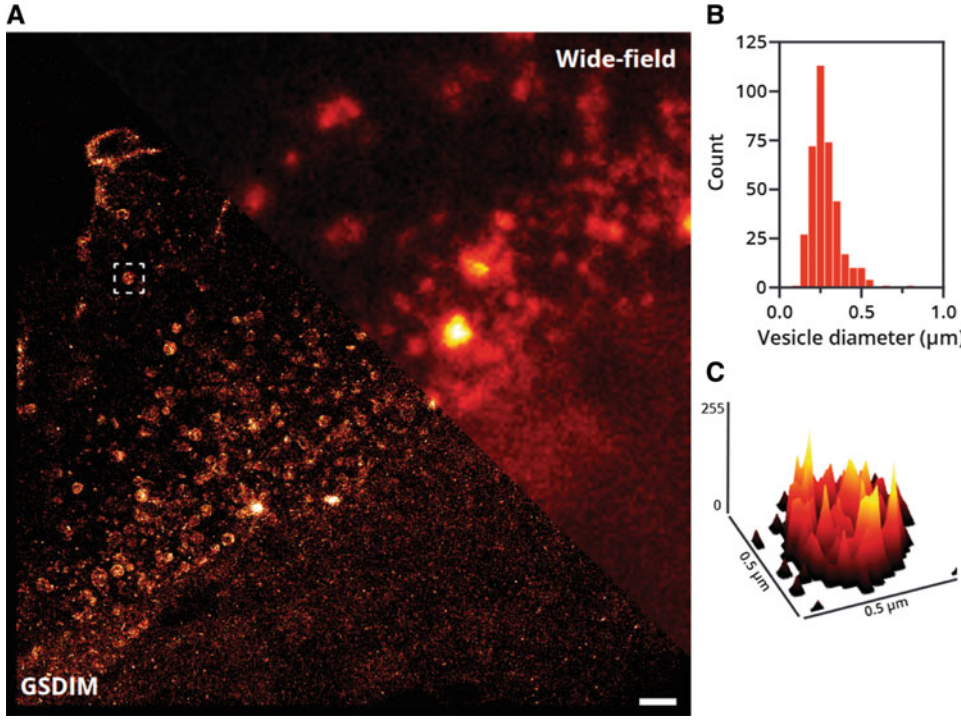

Fig. 1 *Super-resolution imaging of photosensitizer-carrying liposomes*. Bone osteosarcoma (U2OS) cells were seeded on ultraclean coverslips, exposed to 50 μM of NBD-tagged ETLs for 30 min, and processed for GSDIM microscopy as described in this protocol. NBD was visualized using a Leica SR GSD equipped with a Leica PL APO 160×/1.40 oil immersion objective and mounted with a sCMOS pco.edge42 camera. 20,000–50,000 frames were acquired per image and processed using the ImageJ plug-in ThunderStorm [14]. (**a**) Comparison of super-resolution and widefield image. Scale bar represents 1 μm. (**b**) Histogram of vesicle diameter. (**c**) Histogram of square region of interest depicted in **a**

References

1. Kruger CA, Abrahamse H (2018) Utilisation of targeted nanoparticle photosensitiser drug delivery systems for the enhancement of photodynamic therapy. Mol Basel Switz 23. https://doi.org/10.3390/molecules23102628
2. Hong EJ, Choi DG, Shim MS (2016) Targeted and effective photodynamic therapy for cancer using functionalized nanomaterials. Acta Pharm Sin B [Internet] 6:297–307. [cited 2020 Dec 8]. https://doi.org/10.1016/j.apsb.2016.01.007
3. Hell SW, Kroug M (1995) Ground-state-depletion fluorescence microscopy: a concept for breaking the diffraction resolution limit. Appl Phys B [Internet] 60:495–497. [cited 2020 Dec 8]. https://doi.org/10.1007/BF01081333
4. Fölling J, Bossi M, Bock H, Medda R, Wurm CA, Hein B, Jakobs S, Eggeling C, Hell SW (2008) Fluorescence nanoscopy by ground-state depletion and single-molecule return. Nat Methods 5:943–945. https://doi.org/10.1038/nmeth.1257
5. Bardhan R, Lal S, Joshi A, Halas NJ (2011) Theranostic nanoshells: from probe design to imaging and treatment of cancer. Acc Chem Res 44:936–946. https://doi.org/10.1021/ar200023x
6. Silva AKA, Kolosnjaj-Tabi J, Bonneau S, Marangon I, Boggetto N, Aubertin K, Clément O, Bureau MF, Luciani N, Gazeau F, Wilhelm C (2013) Magnetic and photoresponsive theranosomes: translating cell-released vesicles into smart nanovectors for cancer therapy. ACS Nano 7:4954–4966. https://doi.org/10.1021/nn400269x

7. Sharonov A, Hochstrasser RM (2006) Wide-field subdiffraction imaging by accumulated binding of diffusing probes. Proc Natl Acad Sci U S A 103:18911–18916. https://doi.org/10.1073/pnas.0609643104
8. Kuo C, Hochstrasser RM (2011) Super-resolution microscopy of lipid bilayer phases. J Am Chem Soc 133:4664–4667. https://doi.org/10.1021/ja1099193
9. Liu J-X, Xin B, Li C, Xie N-H, Gong W-L, Huang Z-L, Zhu M-Q (2017) PEGylated perylenemonoimide-dithienylethene for super-resolution imaging of liposomes. ACS Appl Mater Interfaces 9:10,338–10,343. https://doi.org/10.1021/acsami.6b15076
10. Xu H, Chen B, Gong W, Yang Z, Qu J (2020) Nanoliposomes Co-encapsulating photoswitchable probe and photosensitizer for super-resolution optical imaging and photodynamic therapy. Cytom Part J Int Soc Anal Cytol 97:54–60. https://doi.org/10.1002/cyto.a.23864
11. Nahidiazar L, Agronskaia AV, Broertjes J, van den Broek B, Jalink K (2016) Optimizing imaging conditions for demanding multi-color super resolution localization microscopy. PloS One 11:e0158884. https://doi.org/10.1371/journal.pone.0158884
12. Broekgaarden M, de Kroon AIPM, van Gulik TM, Heger M (2014) Development and in vitro proof-of-concept of interstitially targeted zinc- phthalocyanine liposomes for photodynamic therapy. Curr Med Chem 21:377–391
13. Broekgaarden M, Weijer R, Krekorian M, IJssel B, Kos M, Alles LK, Wijk AC, Bikadi Z, Hazai E, Gulik TM, Heger M (2016) Inhibition of hypoxia-inducible factor 1 with acriflavine sensitizes hypoxic tumor cells to photodynamic therapy with zinc phthalocyanine-encapsulating cationic liposomes. Nano Res 6:1639–1662. https://doi.org/10.1007/s12274-016-1059-0
14. Ovesný M, Křížek P, Borkovec J, Svindrych Z, Hagen GM (2014) ThunderSTORM: a comprehensive ImageJ plug-in for PALM and STORM data analysis and super-resolution imaging. Bioinforma Oxf Engl 30:2389–2390. https://doi.org/10.1093/bioinformatics/btu202