

# A Review of Labeling Approaches Used in Small Extracellular Vesicles Tracing and Imaging

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**Abstract:** Small extracellular vesicles (sEVs), a subset of extracellular vesicles (EVs) originating from the endosomal compartment, are a kind of lipid bilayer vesicles released by almost all types of cells, serving as natural carriers of nucleic acids, proteins, and lipids for intercellular communication and transfer of bioactive molecules. The current findings suggest their vital role in physiological and pathological processes. Various sEVs labeling techniques have been developed for the more advanced study of the function, mode of action, bio-distribution, and related information of sEVs. In this review, we summarize the existing and emerging sEVs labeling techniques, including fluorescent labeling, radioisotope labeling, nanoparticle labeling, chemical contrast agents labeling, and label-free technique. These approaches will pave the way for an in-depth study of sEVs. We present a systematic and comprehensive review of the principles, advantages, disadvantages, and applications of these techniques, to help promote applications of these labeling approaches in future research on sEVs.

**Keywords:** small extracellular vesicles, label, image, tracking, in vivo imaging

## Introduction

Extracellular vesicles (EVs) are a collective term for particles wrapped by lipid bilayers naturally released from cells that cannot self-replicate. It includes several subtypes, with microvesicles, exosomes, and apoptotic vesicles being major subtypes.<sup>1</sup> Exosomes, also known as intraluminal vesicles (ILVs), are small extracellular vesicles (sEVs) formed via the endosomal pathway and range from 30 to 200 nm in diameter. They contain a series of membrane-associated high-order oligomeric protein complexes that display significant molecular heterogeneity.<sup>2</sup> Although sEVs have been discovered for over 80 years, they were initially considered only as metabolic waste products of cells until 2007, when Valad et al<sup>3</sup> discovered that mRNA and microRNA could be transported to other receptor cells via sEVs and perform corresponding physiological functions. Since then, researchers have started to study sEVs in depth, and their biogenesis and physiological functions have started to be discovered continuously.

sEVs carrying specific proteins, lipids, nucleic acids, and glycoconjugates are part of an intercellular signaling network in multicellular tissues. They mediate multiple activities such as signaling and molecular transmission to other cells.<sup>2,4</sup> sEVs can be secreted by almost all types of cells and have been identified in body fluids such as plasma, urine, semen, saliva, bronchial fluid, cerebrospinal fluid, breast milk, serum, amniotic fluid, synovial fluid, tear fluid, lymphatic fluid, bile, and gastric acid.<sup>5</sup> This pathway of sEVs transport plays an essential role in many aspects of human health and disease, including development, immunity, tissue homeostasis, cancer, and neurodegenerative diseases. In addition, viruses select the sEVs biogenesis pathway to assemble infectious particles and establish host compatibility.<sup>2</sup> Because of their known physiological and pathological functions, their clinical application as biomarkers, therapeutic agents, and therapeutic molecular carriers are considered widely promising and have yielded notable results.<sup>4</sup>

The formation, release, recycling, uptake, and content delivery of sEVs have been critical aspects of sEVs research, and the translation and application of research results are still in their infancy. The study of these problems requires the help of adequate sEVs labeling and tracer imaging technique, and the appropriate labeling technique can help researchers to investigate the biological properties of sEVs in depth. Therefore, effective *in vivo* and *in vitro* sEVs labeling techniques are essential. This paper provides a review of sEVs labeling techniques.

## Membrane Fluorescent Dye Labeling Technique

### Lipophilic Fluorescent Dyes

#### Carbonaceous Dyes

Carbonaceous dyes are a class of lipophilic fluorescent dyes that can be used to stain cell membranes and other lipid-soluble biological structures, including membranes of sEVs. Their fluorescence intensity is greatly enhanced when bound to lipid membranes, and these dyes have high quenching constants and long excited state lifetimes. Once the lipid bilayer is stained, these dyes diffuse throughout the whole membrane and can be applied to the entire membrane structure of sEVs at optimal concentrations. Common carbocyanine dyes include DiI C18(5) (DiI D) ( $\lambda_{ex}/\lambda_{em}=646/663$  nm), DiI C18(3) (DiI) ( $\lambda_{ex}/\lambda_{em}=551/569$  nm), DiOC18(3) (DiO) ( $\lambda_{ex}/\lambda_{em}=483/501$  nm), and DiI C18(7) (DiR) ( $\lambda_{ex}/\lambda_{em}=754/778$  nm). DiI and DiO are widely used for *in vitro* tracing of sEVs due to their very weak fluorescent signal before entering the membrane and the intense signal after entering the membrane structure. Yu et al<sup>6</sup> used DiI to identify the uptake of sEVs by bacteria in their study of the effect of different types of sEVs on bacterial growth. Murdica et al<sup>7</sup> used DiO-labeled endometrial cells and found that their derived sEVs enhanced the ability of sperm to capacitate and acrosome react. And discovering an emerging way for female genitourinary tract cells to interact with sperm. On top of DiI and DiO, DiD and DiR have a lower background fluorescence, while their staining is more stable, and almost no staining transfer between sEVs occurs. More importantly, its longer Near Infrared (NIR) wavelength gives it better results *in vivo* experiments due to the significant reduction in autofluorescence of animals at higher wavelengths.<sup>8</sup> Based on this property, this dye has been used in both *in vivo* and *in vitro* studies of sEVs.<sup>8-10</sup> Santelices et al<sup>9</sup> detailed the labeling procedure of sEVs obtained from *in vitro* culture using DiR. Wen et al<sup>10</sup> used DiD to achieve *in vivo* imaging of MSC-EVs in mice and verified the accumulation of MSC-EVs at radiation-damaged sites in mice.

#### FM Styryl Dyes

FM dyes are a type of lipophilic styrene-based compound with bipolarity. Hydrophilic grouping effectively avoids the occurrence of membrane penetration staining and intercellular cross-staining. Thus, they are widely used in experiments related to membrane labeling and vesicle formation. FM dyes include FM 1-43 (N-(3-Triethylammoniumpropyl)-4-(4-(dibutylamino) styryl)pyridinium dibromide) ( $\lambda_{ex}/\lambda_{em}=510/626$  nm) and FM 4-64(N-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide) ( $\lambda_{ex}/\lambda_{em}=558/734$  nm). Wolf et al<sup>11</sup> used FM 4-64-labeled bovine milk sEVs in human colon cancer cells Caco-2 and rat small intestinal cells IEC-6 to elucidate the mechanism by which intestinal transport of bovine milk sEVs is mediated by endocytosis.

#### PKH Dyes

PKH dyes are another class of lipophilic dyes commonly used for sEVs tracing, invented by Paul Karl Horan<sup>12</sup> and hence the name. Their two long lipophilic tails can be inserted into the lipid bilayer structure, while the fluorescent groups emit fluorescence outside the lipid membrane. Commonly used PKH dyes include PKH26 ( $\lambda_{ex}/\lambda_{em}=551/567$  nm) and PKH67 ( $\lambda_{ex}/\lambda_{em}=490/502$  nm). PKH dyes can also be used in *in vivo* and *in vitro* studies of sEVs.<sup>13-15</sup> Reclusa et al<sup>13</sup> compared PKH26 with PKH67 for labeling imaging during phagocytosis of sEVs in lung cancer cells and observed that better image intensity and contrast were achieved using PKH67 for labeling sEVs. Unfortunately, the underlying causes for this phenomenon have not been extensively investigated in the corresponding article. Pollalis et al,<sup>15</sup> on the other hand, exploited the *in vivo* imaging capability of PKH26 and found the targeting of murine retinal sEVs delivery capability. Although PKH series dyes are now widely used for sEVs labeling, there is a growing consensus that the PKH series may not be suitable, Dehghani et al<sup>16</sup> found that using PKH to label sEVs severely affects the particle size of sEVs. Also, the

aggregation effect of PKH dyes is very significant. It can be taken up by cells, leading to false positives, which is detrimental to the analysis of the results.<sup>17</sup> The use of PKH dyes is worth considering during future sEVs studies.

### Nobel Lipophilic Fluorescent Dye

To overcome the typical defects of traditional dyes like PKH, Shimomura et al<sup>18</sup> reported a class of lipophilic fluorescent dyes called Mem ( $\lambda_{ex}/\lambda_{em}$  = 494–652 nm/512–672 nm). Mem synthesis contains glutamic acids to avoid probe penetrating and aggregation. They were verified by experimenters to show that they did not affect the size of sEVs and did not form aggregation. The CellMask<sup>TM</sup> ( $\lambda_{ex}/\lambda_{em}$ =522–649 nm/535–666 nm) series of dyes is a commercially available lipophilic dye whose most remarkable feature is slow internalization and can be used for coloration after cell fixation. However, the detergent will destroy the structure of CellMask<sup>TM</sup>. Therefore, they cannot be used with probes requiring permeabilization. El-Andaloussi et al<sup>19</sup> employed CellMask<sup>TM</sup> for tracing sEVs to monitor their uptake in an experimental method, they introduced for delivering siRNA in vitro using sEVs. In 2019, Collot et al<sup>20</sup> invented a series of six fluorescent dyes called MemBright<sup>TM</sup> (Figure 1A) with a long wavelength range ( $\lambda_{ex}/\lambda_{em}$ =499–689 nm/506–713 nm, Figure 1B). These new membrane probes are based on zwitterionic anchors and six dialkyne cyanine fluorophores. Due to its amphiphilicity, it forms soluble aggregations in aqueous media and quenches spontaneously until it dissociates in contact with lipids and emits fluorescence (Figure 1C). Its working concentration is exceptionally low, and nanomolar concentration dye can stain plasma membrane structures exactly. Compared to conventional lipophilic dyes, it has the advantages of simpler handling, greater specificity, lower working concentration, and no cytotoxicity. It could be used with live/fixed cells for immunostaining and is compatible with different fluorescence imaging techniques such as long-term, two-photon, tissue, and super-resolution imaging. Subsequently, Hyenne et al<sup>21</sup> verified the feasibility of tracking circulating tumor EVs in zebrafish embryos in vivo (Figure 1D). The following year, Boyer et al<sup>22</sup> also applied MemBright<sup>TM</sup> dye for labeling and tracing sEVs in a study that confirmed that endothelium-derived sEVs regulate the phenotype of vascular smooth muscle cells.

### Membrane Analog

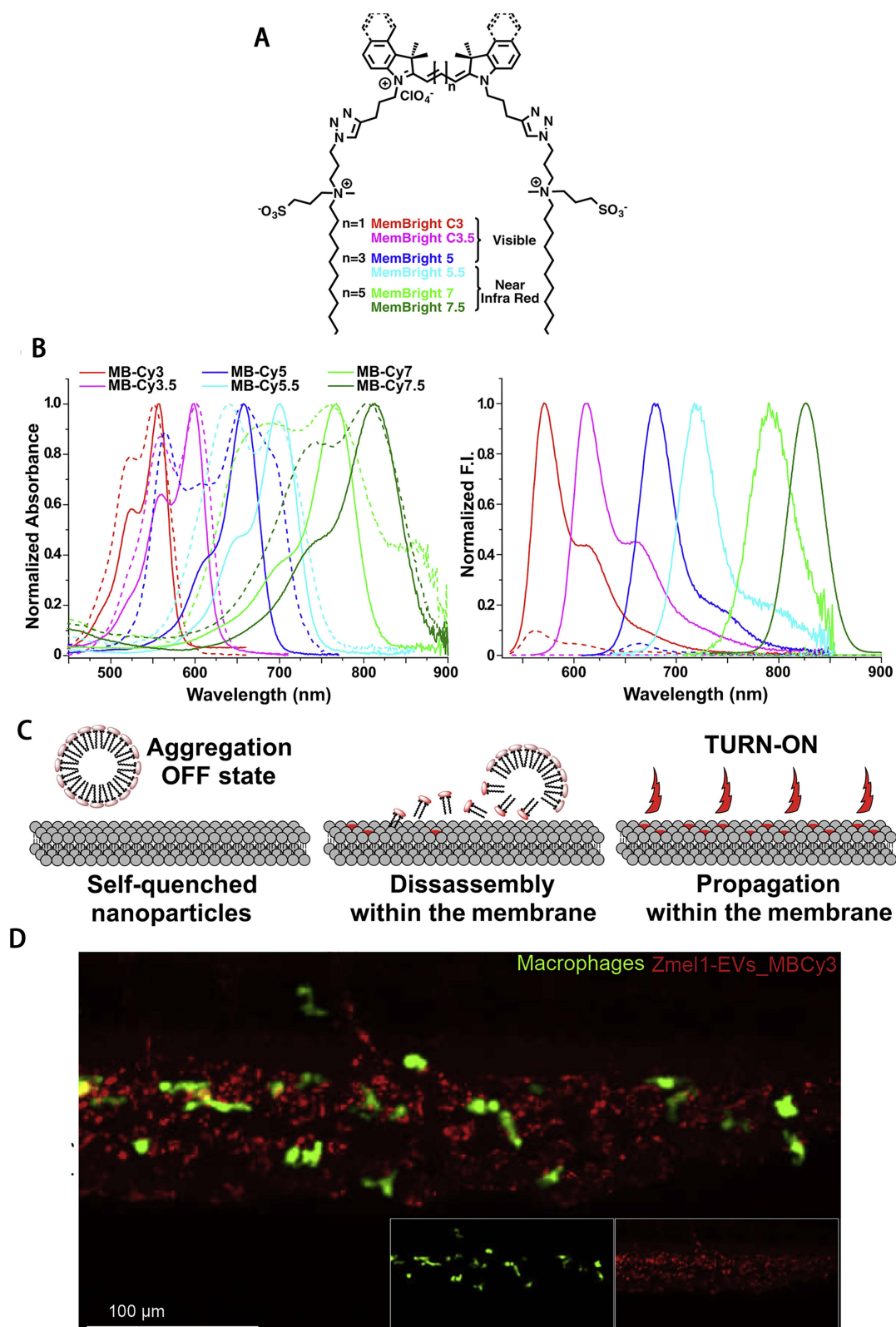
The effective staining labeling of sEVs membranes can also be formed by fluorescently labeled analogs of lipid membrane components. Verderio et al<sup>23</sup> reported a technique for successful labeling of NBD-C6-HPC, a phosphorylcholine analog, onto bone marrow microvesicular membranes. Similarly, Hu et al<sup>24</sup> reported a cholesterol analog labeling dye called 3-NBD-cholesterol. It is verified in their experiments that they could form effective labels for sEVs by co-culturing labeled platelet sEVs with phagocytes and HEK293 cells and can be detected by fluorescence microscopy and flow cytometry. Like the carbocyanine dyes, NBD ( $\lambda_{ex}/\lambda_{em}$ =463/536 nm) also forms effective fluorescent labeling only after entering the membrane structure, avoiding high background, misleading labeling, and other phenomena.

### Fluorescent Lectins

Lectins are a class of naturally occurring proteins with hemagglutinating activity and sugar specificity. Because of its ability to bind glycoconjugates and glycoproteins in cell membrane structures, it is widely used as a tool for membrane and cell research.<sup>25</sup> Combining lectins with fluorescent moieties for labeling cell membranes is widely used. Wheat germ agglutinin (WGA), which binds to N-acetylglucosamine and N-acetylneuraminic acid (sialic acid), is the most widely used. Lennon et al<sup>26</sup> used WGA-647 ( $\lambda_{ex}/\lambda_{em}$ =650/670 nm) to label pancreatic cancer EVs captured by Ab cetuximab because of their overexpression of epidermal growth factor receptor 1 (EGFR) and successfully applied quantitative single-molecule localization microscopy (qSMLM) to image them.

### Self-Quenched Probe for Surveying Membrane Fusion

Octadecyl Rhodamine B Chloride (R18) ( $\lambda_{ex}/\lambda_{em}$ =560/590 nm) is a lipophilic probe that binds to membranes with its fluorescent moiety located in the aqueous phase layer and the alkyl tail extending into the inner lipid layer. R18 fluorescence is quenched at high concentrations and is gradually released once the concentration is diluted. This feature



**Figure 1** (A) Structure of the MemBright™ markers. (B) Absorption and emission spectra of MemBright™ probes (200 nM) in the absence (dashed lines) or presence of DOPC vesicles. (C) Turn-on mechanism of the MemBright™ probes. (A)-(C) Reprinted from *Cell Chem Biol*, 26(4), Collot M, Ashokkumar P, Anton H, et al. MemBright: a family of fluorescent membrane probes for advanced cellular imaging and neuroscience. 600–614 e7, Copyright (2019), with permission from Elsevier.<sup>20</sup> (D) Confocal images of MemBright-Cy3 labeled Zmel1 EVs 3 hpi in Tg (mpeg1: GFP) (macrophage specific expression). Reprinted from *Dev Cell*, 48(4), Hyenne V, Ghoroghi S, Collot M, et al. Studying the fate of tumor extracellular vesicles at high spatiotemporal resolution using the zebrafish embryo. 554–572 e7, Copyright (2019), with permission from Elsevier.<sup>21</sup>

makes it a probe for membrane fusion studies. Tian et al<sup>27</sup> used this feature to distinguish between two different modes of sEVs uptake: endocytosis and membrane fusion.

Traditional lipid membrane dyes have been widely used as tracing markers in sEVs research due to their early application, relatively simple experimental procedures, low cost, strong stability, and long half-life. However, as their practical applications have progressed, more and more issues have emerged. For example, (i) current methods for isolating sEVs struggle to remove lipid contaminants, inevitably leading to nonspecific staining. (ii) Lipophilic dyes, due to their molecular characteristics, easily form aggregates that can affect the interpretation of experimental results. This situation is particularly severe when using samples obtained through ultracentrifugation. (iii) Lipid membrane dyes used for labeling sEVs may impact membrane fusion with cells, the physiological properties of the membrane, the texture of the membrane, and the size of sEVs. (iv) Due to dye transfer and their much longer half-life compared to the lifespan of sEVs, there can be an impact on the study of sEVs outcomes within cells. Some researchers recommend avoiding the use of membrane dyes for labeling sEVs of plasma samples obtained through ultracentrifugation. Additionally, sEVs labeling experiments should involve thorough washing to remove unbound dye and require careful experimental design with appropriate controls to correctly interpret the results. Considering the poor performance of PKH dyes in experiments, their use for sEVs labeling needs to be carefully considered. Overcoming the limitations of traditional dyes, novel dyes with self-quenching, small molecular size, and high affinity should be of interest and consideration for researchers.<sup>16,17,28–33</sup>

## Bio-Conjugation Labeling Technique

### Amine-Reactive Dyes

Amine-reactive dyes are a class of tracer dyes that are labeled by amide reactions. Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) is the most commonly used amine-reactive dye for sEVs tracing. CFDA-SE ( $\lambda_{\text{ex}}/\lambda_{\text{em}}=500\text{ nm}/520\text{ nm}$ ) CFSE can easily penetrate cell membranes, covalently bind to intracellular proteins in living cells, and release green fluorescence after hydrolysis. The succinimidyl ester reacts with the intracellular amino group to form a stable fluorescent conjugate that can be fixed with an aldehyde fixator. Due to the labeling located inside the cells and the sEVs, it does not affect the size and function of sEVs, and the labeling is effective for several weeks.<sup>16,34</sup> Radnaa et al<sup>35</sup> labeled sEVs containing high mobility group box 1 protein (HMGB1) with CFDA-SE to study the mechanism of this sEVs molecule. Notably, the inherent properties of carboxyfluorescein, whose fluorescence intensity is too weak in an acidic environment, prevent its application for monitoring the exocytosis of sEVs in the endosomal pathway.<sup>36</sup> Given that the defect of CFDA-SE, Zhou et al<sup>37</sup> developed a novel amine-reactive dye called ExoTracker ( $\lambda_{\text{ex}}/\lambda_{\text{em}}=550\text{ nm}/670\text{ nm}$ ), which is conjugated to the sEVs membrane by amide reaction and undergoes structural changes in this process to form a fluorescent label. Its pH characteristics are opposite to those of CFDA-SE. It has a better fluorescent ability in acidic environments, thus complementing CFDA-SE. Besides, Tian et al<sup>38</sup> used an amine-reactive dye called TAMRA-NHS (carboxytetramethylrhodamine succinimidyl ester) ( $\lambda_{\text{ex}}/\lambda_{\text{em}}=553\text{ nm}/575\text{ nm}$ ), whose NHS binds to r membrane proteins of sEVs to achieve a labeling tracer effect when studying the fate of protein components of sEVs being uptake by recipient cells.

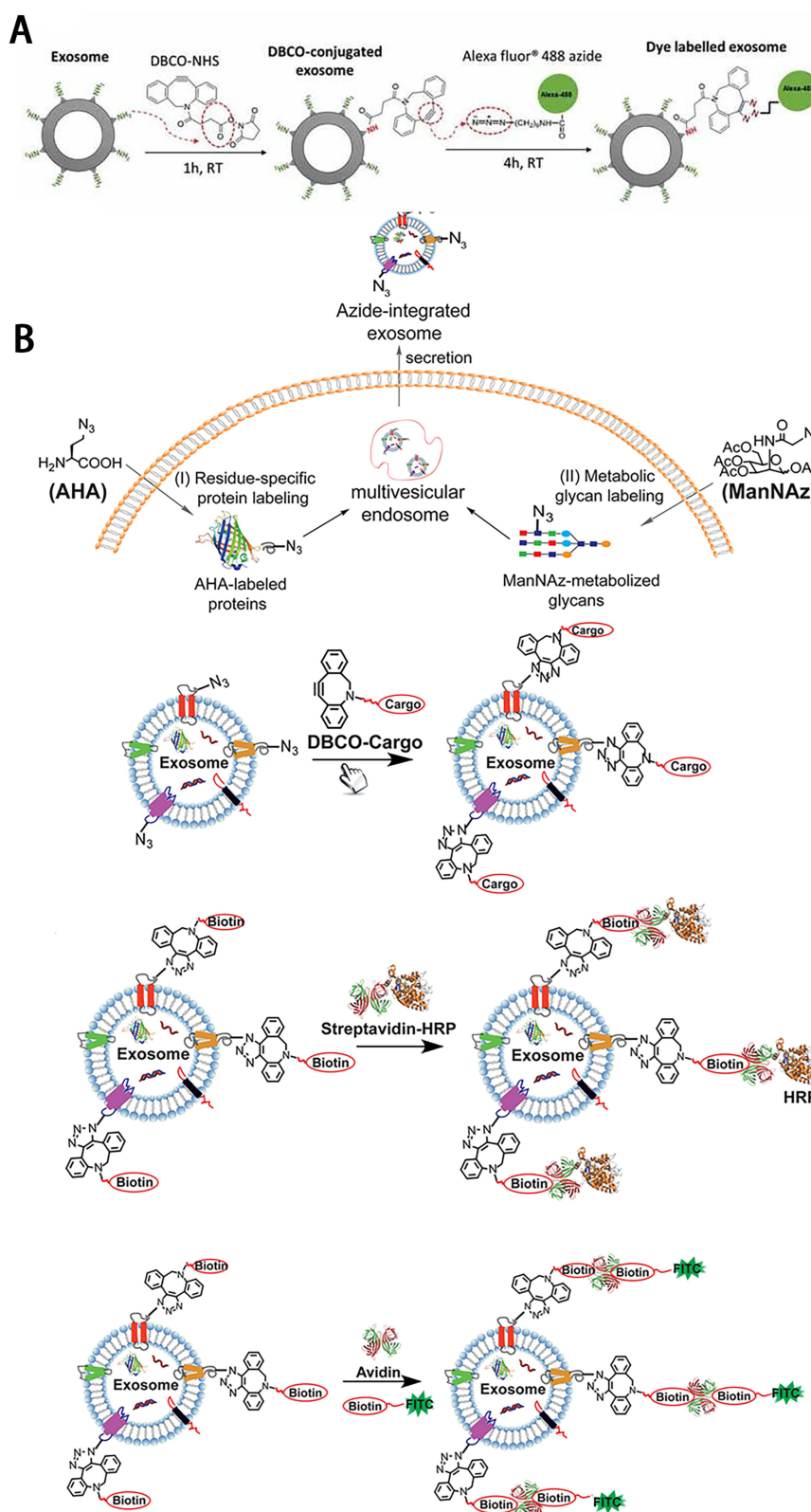
### Thiol-Reactive Dyes

Thiol-reactive dyes are a class of tracer dyes that label sEVs by combining fluorescent groups with maleimide. This organic compound can combine with thiol groups in tetraspanins on the sEVs.<sup>39</sup> Roberts-Dalton et al<sup>40</sup> used Alexa488 ( $\lambda_{\text{ex}}/\lambda_{\text{em}}=494\text{ nm}/517\text{ nm}$ ) combined with maleimide to form a novel fluorescent dye to label sEVs and then uptake by Hela cells. They found that sEVs are uptake by clathrin-independent endocytosis and ultimately delivered to the lysosome. Labelling of EVs in this way did not influence their size and had no effect on their abilities.

### Azide-Alkyne-Cycloaddition Labeling Technique

The azide-alkyne-cycloaddition reaction is a click chemical reaction between the azide and the strained alkyne ring, which is catalyzed by the copper to form a triazole bond. A copper-free reaction method was also developed for biological research.<sup>41</sup> There are three primary forms of this labeling method: i) Direct labeling of isolated sEVs with chemicals containing azides and alkynes. Xu et al<sup>42</sup> successfully labeled PANC-1 Exo through the reaction between the





**Figure 2 (A)** Scheme of the fluorescence labelling method of sEVs surface using copper-free click chemistry with AlexaFluor® 488 (AF488)-azide. Reprinted from Xu L, Faruqi FN, Liam-Or R, et al. Design of experiment (DoE)-driven and uptake studies of exosomes for pancreatic cancer delivery enabled by copper-free click chemistry-based labelling. *J Extracell Vesicles*. 2020;9(1):1779458. © 2020 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group on behalf of The International Society for Extracellular Vesicles. Creative Commons.<sup>42</sup> **(B)** Combination of the metabolic labeling of newly synthesized proteins or glycan/glycoproteins of exosome-secreting cells with chemically active azide groups and biorthogonal click conjugation to modify and functionalize sEVs. Reprinted from Wang M, Altinoglu S, Takeda YS, Xu Q. Integrating Protein Engineering and Bioorthogonal Click Conjugation for Extracellular Vesicle Modulation and Intracellular Delivery. *PLoS One*. 2015;10(11):e0141860. Creative Commons.<sup>43</sup>

N-hydroxysuccinimidyl (NHS) group of dibenzobicyclooctyne (DBCO) and amine groups of lysine residues on sEVs surface proteins (Figure 2A). ii) Supplementing sEVs-producing cells with azide- or alkyne-containing amino acids to produce azide- or alkyne-containing proteins by the cells' own biosynthesis and adding the corresponding compound with the labeling group to label the cells and their sEVs. iii) Supplementing sEVs-producing cells with azide- or alkyne-containing amino acid glycans/proteoglycans to generate membrane surface glycans containing azide or alkyne. Then add the corresponding compound with the labeling group to label the cells and their sEVs. Wang et al<sup>43</sup> used a residue-specific protein labeling strategy. They co-cultured sEVs-producing cells with L-azidohomoalanine (AHA), an azide-bearing amino acid analog of methionine and tetra-acetylated N-azidoacetyl-D-mannosamine (ManNAz), an azidosugar that can be metabolized into sialic acid. Unnatural azides were introduced into sEVs through protein and sugar metabolism. Then, the addition of DBCO with reporting groups to the culture system achieved valid labeling of producing sEVs (Figure 2B). Because of the relevance of this labeling technique for glycan metabolism at the cell membrane surface, this technique has also been used for glycan imaging and sEVs surface glycan analysis.<sup>44,45</sup> Based on the high flexibility of click chemistry, different labeling types can be easily achieved by substituting reporter moieties and enabling further modification of the composition and functionality of sEVs. Providing novel, powerful tools to study the roles of sEVs in biology and expand the biomedical potential of sEVs.

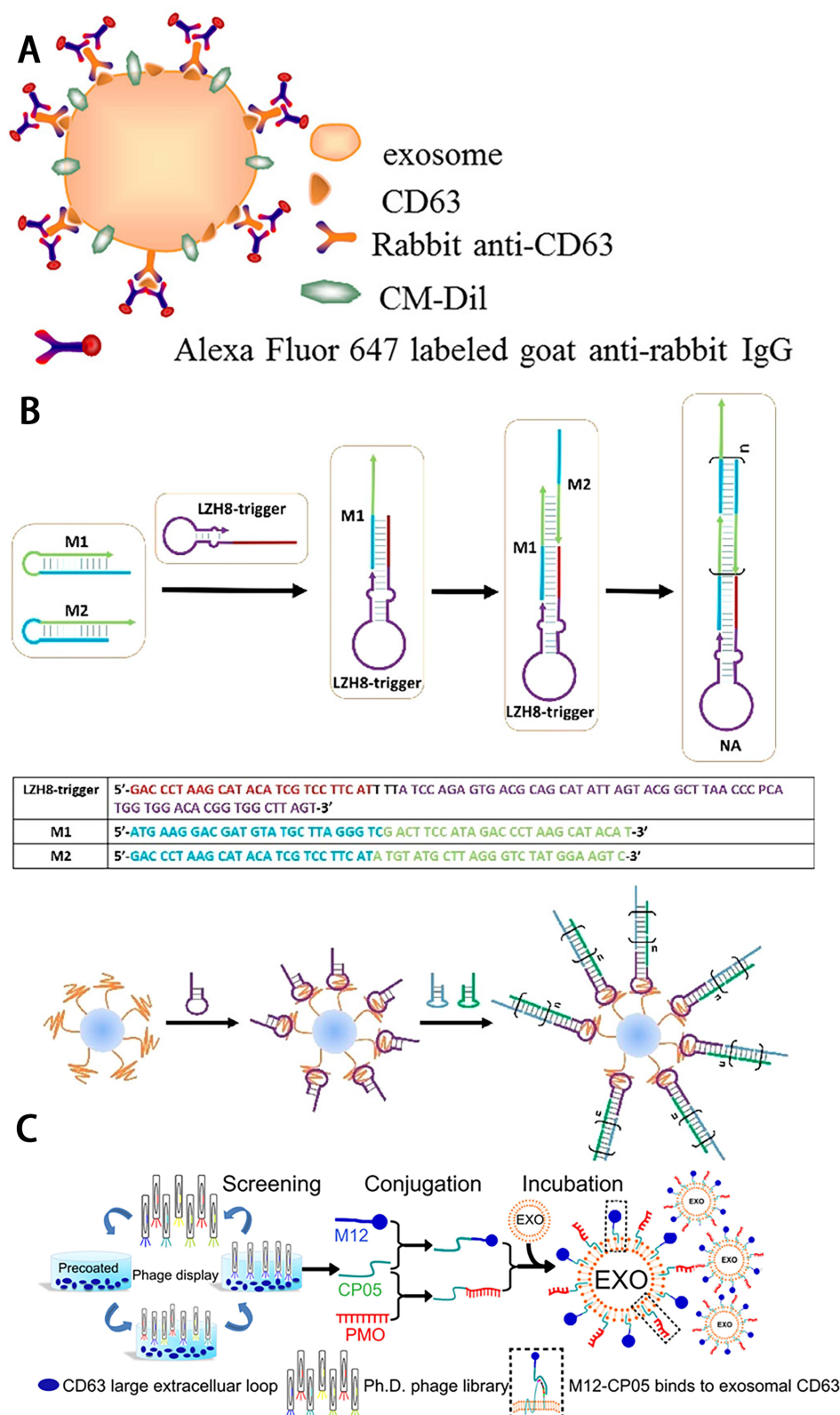
## Non-Covalent Labeling Technique

### Immunolabeling

Immunolabeling is also a commonly used labeling technique. The main principle is to label the sEVs target protein with the corresponding primary antibody and then bind the primary antibody with the secondary antibody coupled with a fluorescent group. Then the sEVs are valid labeled. Tetraspanins are specific sEVs membrane proteins that are the most commonly used primary antibody-binding proteins. Chen et al<sup>46</sup> labeled sEVs secreted by breast cancer cells using secondary antibodies coupled to Alexa Fluor 647 with primary antibodies recognizing CD63 proteins (Figure 3A). Besides Tetraspanins, sEVs-specific proteins such as TSG101 and HSP70<sup>47</sup> or target sEVs-specific proteins like HER-2<sup>46</sup> can also be labeled as primary antibody-binding proteins. Mondal et al<sup>47</sup> took glioblastoma (GB) cell-line KW10 as an example to describe immunofluorescence labeling techniques for sEVs membrane-specific proteins CD63, TSG101 and calnexin in detail. Based on antigen-antibody reactions, immunolabeling exhibits strong specificity, allowing effective discrimination of different sources of sEVs or different subpopulations of sEVs as needed. However, this method also has some drawbacks. Due to the small diameter of sEVs, the binding of a large number of antibodies and labeled proteins on the surface of sEVs will increase the size of sEVs and obscure other functional receptors on their surface, thus seriously affecting their physicochemical properties and biological functions.

### Labeling by Aptamers

The aptamer is a short single-stranded nucleic acid molecule typically generated by the systematic evolution of ligands by exponential enrichment (SELEX). The desired aptamers can then be selected in the synthetic library. The aptamer can specifically bind to target molecules in a similar manner to antigen-antibody binding.<sup>50</sup> Wan et al<sup>48</sup> reported an aptamer-based nanolabeling technique on the surface of sEVs, which selected the HepG2 cell-specific binding aptamer LZH8, and achieved specific labeling of HepG2 sEVs by hybridization chain reaction with two nucleic acid monomers to form a switch structure and then added fluorescein isothiocyanate (FITC) to achieve fluorescent labeling of HepG2 sEVs (Figure 3B). The authors claimed that this technique could reach different types of labeling by changing the labeling group. Zhu et al<sup>44</sup> reported a dual labeling method for aptamer and orthogonal chemical labeling. They achieved dual labeling of exoPD-L1 on sEVs surfaces, respectively, through orthogonal chemistry labeling by glycan metabolism with alkyne DBCO-Cy5 and PD-L1-specific aptamer with Cy3. They first demonstrated that glycosylation of exoPD-L1 is the structural basis for exoPD-L1/PD-1 interaction and inhibition of CD8+T cell proliferation. It provides a theoretical basis for immunotherapy. Compared with antigen-antibody reactions, aptamer molecules are smaller, have higher biocompatibility, and can effectively avoid the impact of labeling on sEVs physicochemical properties and biological functions.<sup>51</sup>



**Figure 3** (A) Schematic illustration of indirect IF labeling of CD63 and sEVs membrane stained with CM-Dil. Reprinted with permission from Chen C, Zong S, Wang Z, et al. Imaging and Intracellular Tracking of Cancer-Derived Exosomes Using Single-Molecule Localization-Based Super-Resolution Microscope. *ACS Appl Mater Interfaces*. 2016;8 (39):25825–25833. Copyright © 2016 American Chemical Society.<sup>46</sup> (B) Schematic of aptamer-based DNA nanoassemblies on the surfaces of sEVs. Reprinted with permission from Wan S, Zhang L, Wang S, et al. Molecular Recognition-Based DNA Nanoassemblies on the Surfaces of Nanosized Exosomes. *J Am Chem Soc*. 2017;139 (15):5289–5292. Copyright © 2017 American Chemical Society.<sup>48</sup> (C) Schematic illustration of screening for sEVs anchor peptides and functionalization on sEVs. Reprinted from Gao X, Ran N, Dong X, et al. Anchor peptide captures, targets, and loads exosomes of diverse origins for diagnostics and therapy. *Sci Transl Med*. 2018;10:444. Creative Commons.<sup>49</sup>



## Labeling by Anchoring CP05 Peptide

CP05 peptide has a strong affinity for the extracellular second ring structure of CD63, an sEVs-specific protein. Hence, it can be used as an effective linkage between the marker molecule and sEVs. Gao et al<sup>49</sup> verified the in vitro tracer ability of CP05 by co-incubating CP05 labeled by Rhodamine with sEVs. They bound AF680-labeled CP05 to various sEVs, demonstrating its in vivo tracer and nonspecifically-binding sEVs properties. They also labeled Rhodamine-labeled M12-CP05, a muscle-targeting peptide, and FITC-labeled NP41-CP05, a nerve-targeting peptide with sEVs, verifying CP05's dual labeling capability and no interaction between the different CP05 markers (Figure 3C). As a short peptide, CP05 has minimal impact on the functionality and size of sEVs. Additionally, its production difficulty is relatively lower compared to monoclonal antibodies, and the subsequent conjugation of molecules to CP05 is simpler.

## Labeling by Electrostatic Interactions

The sEVs surface is considered to be negatively charged overall due to its richness in proteoglycans, sialic acid, and lipids. This allows us to label sEVs by making the target molecule positively charged.<sup>52–54</sup> DPA-SCP is a positively charged aggregation-induced emission luminogens (AIEgens) that can be directly adsorbed to the sEVs membrane surface via electrostatic interaction. Using this technique, Cao et al<sup>55,56</sup> injected DPA-SCP-labeled MSC-EVs into liver-injured or kidney-injured mice and successively observed specific aggregation of MSC-EVs in the injured organs, verifying the in vivo and in vitro labeling ability of AIEgens. The experimental results demonstrate that, apart from slightly altering the membrane potential of sEVs, no significant impact on the size and functionality of sEVs was observed. Additionally, compared to traditional labeling methods such as PKH26 and DiO, it exhibited superior imaging effects.

## Labeling sEVs via Cell-Engineering Technique

Biomarkers such as tetraspanins, heat shock proteins (HSPs), lactadherins, and donor cell-specific proteins like viral envelope protein VSVG<sup>57</sup> are present on the sEVs membrane surface. By integrating the coding sequences of fluorescent proteins or luciferases with those of these proteins through genetic engineering techniques, the corresponding fusion proteins can be constructed to label and trace sEVs under specific conditions.

## Labeling by Fluorescent Protein

The fluorescent protein is a commonly used reporter protein that emits a corresponding fluorescent signal in response to excitation light of a specific wavelength. We can trace the tagged sEVs by constructing a fusion protein from reported fluorescent protein fusions to sEVs proteins. Currently, a fusion protein that binds CD63 to a reporter protein is widely used. Men et al<sup>58</sup> constructed a mouse model expressing CD63-GFP fusion protein and determined the localization of neuron-secreted sEVs in astrocytes. Similarly, Li et al<sup>59</sup> used a CD63-iRFP682 fusion protein to establish a breast cancer cell line that stably expresses sEVs containing this protein. With the exception of the traditional form of fusion proteins, scholars have developed a new type of fusion protein. Fluorescent proteins such as EGFP, tdTomato, and mNeonGreen<sup>60,61</sup> are bound to the palmitoyl group. Then, with the catalyzation by the intrinsic palmitoyl acyltransferase (PAT)<sup>62</sup> of somatic cells, the palmitoyl group is bound to thiol groups on the surface of the sEVs membrane. Then the sEVs are bound by the fluorescent protein.

## Labeling by Bioluminescence

The bioluminescent labeling technique is also called the luciferase labeling technique. Its principle is similar to fluorescent proteins. However, unlike fluorescence imaging, which requires excitation light, luciferases can emit light spontaneously when combined with specific substrates. Common luciferase genes include Fluc, Gluc, Rluc, and Nluc.<sup>63–66</sup> For example, Takahashi et al<sup>64</sup> established a reporter model of a gLuc-LA fusion protein, expressed in mouse melanoma B16BL6 cells. Collecting the sEVs containing fusion protein and injecting them into mice through intravenous injection. They found that sEVs were cleared by macrophage uptake in the liver, spleen, and lung. In their study, Gupta et al<sup>28</sup> compared the labeling effects of five luciferases, NanoLuc, ThermoLuc, Fluc, Super Rluc8, and CBRLuc. The results show higher luminescence

efficiency and more stable labeling of NanoLuc, while ThermoLuc has better performance in *in vivo* experiments due to its NIR emission wavelength. Both of them have higher labeling efficiency for sEVs. Therefore, the authors recommend using NanoLuc for sEVs labeling *in vitro* and ThermoLuc for tracing *in vivo*.

In order to avoid the limitations of using a single fusion protein, two or even multiple-reporter genes could be used simultaneously to label sEVs-producing cells to homogenize the monitoring levels or to monitor multiple components simultaneously.<sup>67,68</sup> Notably, when multiple fusion proteins label the same sEVs-producing cells, different fusion proteins have different loading efficiencies and specificities into the secreted sEVs.<sup>69</sup> Genetically, engineered labeling technique has high specificity, high flexibility, low impact on cells, and high stability compared to traditional membrane dye labeling technique. Although the sEVs of clinical samples are difficult to trace from the source by this technique, its extremely flexible and stable labeling modalities make it promising for the study of sEVs biogenesis, *in vivo* transport, cellular uptake, and eventual fate.

## Radiolabeling

Radioisotope labeling is a classical laboratory labeling technique that has been exploited and applied in sEVs tracing research. Compared with traditional optical labeling techniques, radioactive labeling has high sensitivity and stable imaging performance in tissue imaging, *in vivo* imaging, and even clinical imaging. The commonly used isotopes for sEVs labeling include <sup>131</sup>I, <sup>125</sup>I, <sup>124</sup>I, <sup>111</sup>In, <sup>99m</sup>Tc, <sup>89</sup>Zr, <sup>68</sup>Ga, <sup>64</sup>Cu, and so on. These signals can be detected by single photon emission computed tomography (SPECT) or positron emission tomography (PET) noninvasively. The isolated organs can also be imaged by Computed Tomography (CT) or Magnetic Resonance (MR).<sup>70</sup>

Iodine radioisotopes are an extensively used class of isotopes for labeling. Rashid et al<sup>70</sup> showed the distribution of radioisotope <sup>131</sup>I-labeled tumor sEVs (TDEs) *in vivo* from different cellular origins through SPECT/CT. Morishita et al<sup>71</sup> combined isotopic labeling with genetic engineering techniques to form <sup>125</sup>I labeled sEVs via a streptavidin-lactadherin fusion protein and <sup>125</sup>I labeled biotin and found that the liver predominantly cleared B16BL16-derived sEVs. Royo et al<sup>72</sup> directly labeled sEVs with [<sup>124</sup>I]NaI and monitored them by PET. They demonstrated that sEVs accumulated rapidly in the liver and were present in multiple organs, including the brain.

Despite iodine, metal isotopes are also widely used. Faruqi et al<sup>73</sup> reported two sEVs labeling techniques based on <sup>111</sup>In. One is an endo-labeling technique with tropolone-mediated isotope penetration through the membrane into the sEVs lumen; the other is a membrane labeling technique based on amine-responsive DTPA-anhydride bidirectional binding of sEVs membranes and isotopes. In comparison, the membrane label has better effect and flexibility. <sup>99m</sup>Tc is a more used isotope in the field of sEVs labeling. Gonzalez et al<sup>74</sup> reported a <sup>99m</sup>Tc-based ionic salt (<sup>99m</sup>TcCl<sub>4</sub>) labeling approach. Compared to the previous complex compound labeling like <sup>99m</sup>Tc-HMPAO,<sup>75</sup> (<sup>99m</sup>Tc-tricarbonyl),<sup>76</sup> and [<sup>99m</sup>Tc(CO)<sub>3</sub>(H<sub>2</sub>O)<sub>3</sub>]<sup>+</sup>,<sup>77</sup> ionic salt labeling has a more simpler operation and lower cost, while also ensuring labeling efficiency. Recently, <sup>89</sup>Zr-based sEVs isotope labeling tracer methods have also been reported, including directly labeling sEVs membrane with zirconium-89 (<sup>89</sup>Zr) via amine reactions<sup>78</sup> and endo-labeling with [<sup>89</sup>Zr]Zr(oxinate)<sub>4</sub>.<sup>79</sup> Besides, Jung et al<sup>80</sup> successfully visualized the biodistribution of sEVs in the lymphatic or bloodstream pathways using <sup>68</sup>Ga or <sup>64</sup>Cu-labeled sEVs and demonstrated the higher sensitivity of isotopic labeling, compared with Cy7 fluorescent labeling. Warashina et al<sup>81</sup> also labeled sEVs with <sup>64</sup>Cu via a cross-bridged macrocyclic chelator (CB-TE1A1P) for pharmacokinetic study and determined the contribution of macrophages in the uptake of sEVs by the liver and spleen. In addition, Horgan et al<sup>82</sup> reported an sEVs molecular imaging technique *in vitro* based on Raman metabolic labeling. Deuterium-labeled sEVs are formed by the cell's own metabolism and used for subsequent tracing of cellular uptake for internalization and other studies.

Although radioisotopes labeling in deep tissues has advantages that are difficult to match with conventional labeling methods, its application on sEVs labeling is relatively few. On the one hand, the use of radioactive substances requires large precision instruments and higher operational capability; on the other hand, the use of radioactive substances is under strict surveillance, especially for clinical applications. In addition, when utilizing radiolabeling of sEVs with radioisotope, appropriate chelators or alternative methods should be selected. Direct labeling of sEVs with radioisotopes exhibits drawbacks like detachment after *in vivo* injection or potential impact of radioisotopes and labeling process on the functionality of sEVs. Suitable labeling of media or treatments is a critical issue in the process of radiolabeling. It is also a primary focus for subsequent research endeavors.

## Nanoparticles Labeling

### Metal Nanoparticles Labeling

Metal nanoparticles are excellent and widely used contrast agents for sEVs imaging. Gold is an excellent contrast metal for CT, MRI, and Photoacoustic Imaging (PAI). Popovtzer's team<sup>83–85</sup> developed glucose-encapsulated gold nanoparticles (Glu-GNPs) that could be directly transported into sEVs for CT imaging. They demonstrated that MSC-EVs could cross the blood–brain barrier and accumulate in various types of neuropathic regions through experiments. The results provide new technical support for the diagnosis and treatment of CNS diseases, on the one hand, and fill the gap in CT imaging localization of CNS diseases, on the other hand. Similarly, Lara et al<sup>86</sup> established another method of CT imaging for sEVs using nanogold. They exploited the overexpression of folate receptors on the surface of B16F10 cells and conjugated nanogold with folate to generate AuNP-PEG-FA, which was endocytosed by cells through the folate receptor pathway and then entered their secreted sEVs through biosynthesis. Using this method, they observed that sEVs preferentially accumulated in small lung metastatic tumors and were precisely distributed along the tumor tissue. In addition, gold can also be used as a PAI photothermal contrast agent, combining both PAI contrast and photothermal therapy (PTT).<sup>87</sup> Zhu et al<sup>88</sup> co-incubated tumor cells with gold nanostars to produce a large number of tumor cell-derived stellate plasmonic sEVs (TDSP-Exos) that could be specifically taken up by tumor cells thereby PAI the tumor tissue and kill the tumor cells by PTT.

Superparamagnetic iron oxide nanoparticles (SPIONs), another widely used class of metallic particles, are magnetic particles ranging in size from 5 ~ 200 nm and are mainly used for MRI. Their superparamagnetic character is their most distinctive feature, allowing the movement of SPIONs to designated areas of the body to be controlled by an applied magnetic field.<sup>89</sup> Their low toxicity, high sensitivity, and targeted delivery characteristics achieve better imaging results in the lesion area only at a low dose.<sup>90</sup> First, due to its high biocompatibility, SPIONs can be attached to sEVs by various means such as electroporation, natural cellular uptake, or Bio-Conjugation and carry substances such as other labeling molecules or therapeutic drugs to meet experimental or therapeutic requirements.<sup>91</sup> Busato et al<sup>92,93</sup> labeled 100% of the cells using ultra-small superparamagnetic iron oxide nanoparticles (USPIO, 4–6 nm) and traced their secreted sEVs that retained USPIO labeling and physiological characteristics by MRI. Jia et al<sup>94</sup> loaded SPIONs and curcumin into sEVs conjugated with neuromyelin-1 targeting peptide, which allows simultaneous imaging and therapy of glioma in vitro and in vivo. Zhang<sup>95</sup> loaded sEVs conjugated with rabies virus glycoproteins with SPIONs to synthesize MRI contrast agent with targeting ability to nerve cells and successfully imaged them in vivo.

Some other metals also have the potential as contrast agents, such as manganese<sup>96,97</sup> and gadolinium.<sup>98</sup> Abello et al<sup>98</sup> used lipid gadolinium to label sEVs for MRI and found a sustained accumulation of HUC-MSC sEVs in tumors. However, their application is limited due to the possible biotoxic effects of such metals.<sup>99</sup>

### Quantum Dots Labeling

Quantum dots (QDs) are nanoscale semiconductors that emit light at a specific frequency by applying a particular electric field or optical pressure to this nanosemiconductor material. Compared to conventional organic dyes, they have long-term stability, efficient labeling, and “multiplexing.”<sup>100</sup> Its synthetic customizability and broad excitation spectrum give it an extensive and flexible applicational prospect. Moreover, it has been used for imaging of sEVs in vivo and in vitro for decades.<sup>101</sup> How to label QDs onto target sEVs is the key to QDs labeling techniques, and the related methods have been described previously. For example, sEVs can be labeled by QDs using antigen-antibody or aptamer techniques.<sup>102–105</sup> Alternatively, QDs can be conjugated to sEVs using click chemistry<sup>106</sup> or internalized into sEVs directly using the cells' own uptake and internalization.<sup>107</sup>

The synthetic feature of Nanoparticles (NPs) gives great flexibility to this technique. Some scholars have used specifically synthesized NPs to implement multimodal imaging techniques. For example, Zhao et al<sup>107</sup> synthesized ultra-small Mn magnetically functionalized Ag<sub>2</sub>Se quantum dots (Ag<sub>2</sub>Se@Mn QDs) that can be directly loaded into microvesicles (MVs) by electroporation. The labeled MVs can be traced by NIR and MRI. Bose et al<sup>108</sup> synthesized similar NPs named GION for labeling TEVs for MRI and PTT. Shaikh et al<sup>109</sup> performed CT, MRI, and fluorescence imaging (FLI) multimodal imaging of tumor cells and their derived sEVs using in situ generated iridium iron oxide

nanoclusters (NCs). Cao et al<sup>110</sup> synthesized vanadium carbide quantum dots (V2C QDs) loaded in engineered sEVs for FLI, PAI, MRI multimodal imaging, and tumor-targeted PTT. Tayyaba et al<sup>111</sup> performed CT, MRI, and FLI multimodal imaging of sEVs from Ag-Fe304 NCs biosynthesized in situ from HepG2 cells.

## Chemical Contrast Agent Labeling

In addition to metal NPs, some organic compounds or biomolecular substances also function as contrast agents. For example, chlorin e6 (Ce6) is a photosensitizer that can be used for PAI. Although it is also a commonly used medicine for PTT, its clinical applications are limited by its low plasma concentration and nonspecific toxicity.<sup>112</sup> Jiang et al<sup>113</sup> loaded Ce6 into tumor-derived sEVs to form Ce6-R-Exo that could be targeted accumulated in tumor cells for PAI and PTT. Ferritin heavy chain (FTH1) is an MRI reporter protein.<sup>114</sup> Liu et al<sup>115,116</sup> achieved in vivo and in vitro MRI of target cell-derived sEVs by lentiviral transfection to make the target cells express FTH1.

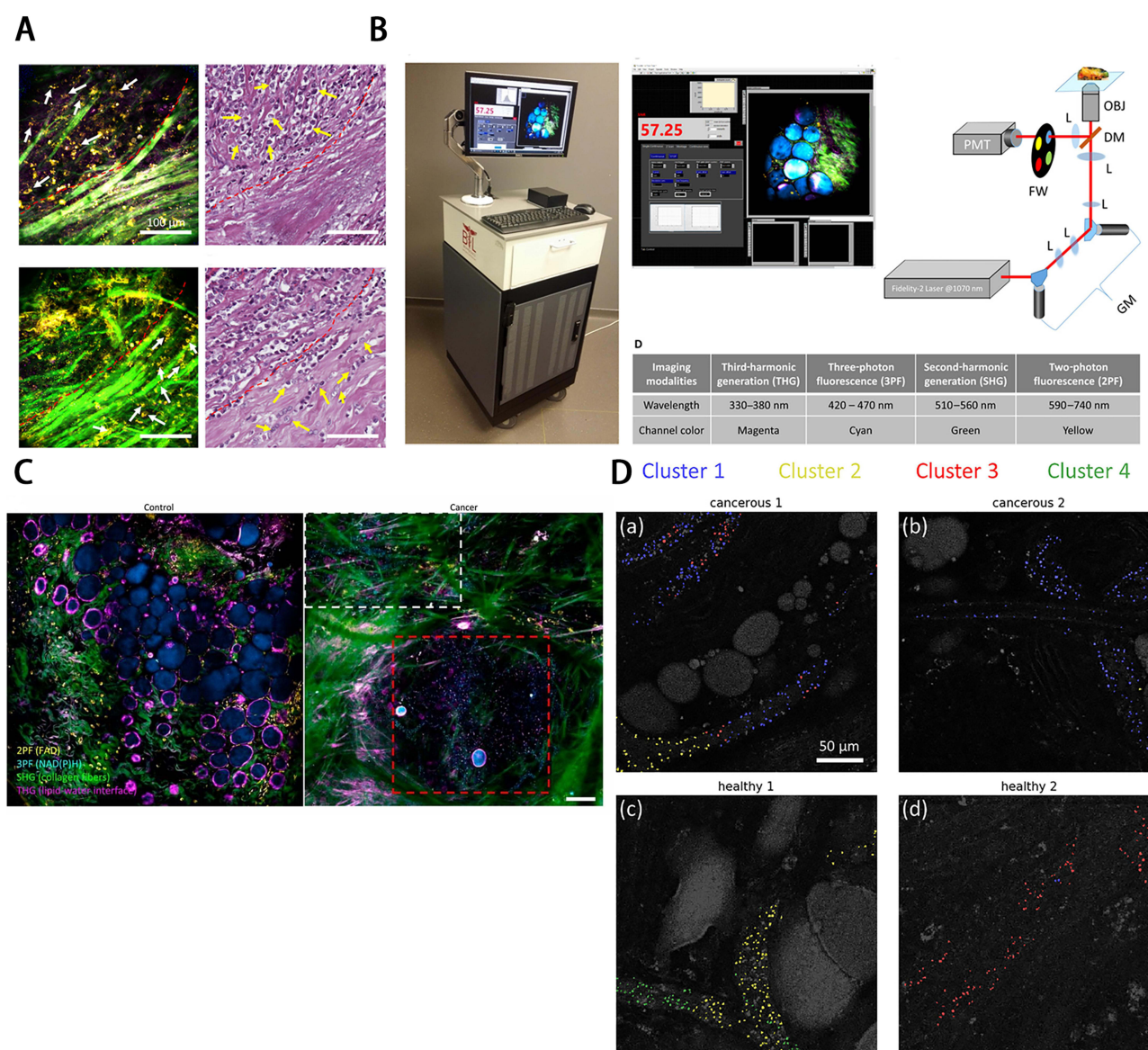
## Label-Free Visualization

The label-free technique, ie, visualizing sEVs without labeling, is usually based on the intrinsic properties of sEVs. The use of label-free technology avoids the influence of sEVs and allows them to be assayed in a state closer to normal physiology, while also reducing the experimental time. Currently, label-free techniques are mainly used for imaging, size analysis, and sorting of sEVs, such as TEM, NTA, DLS, FCM, AFM, and so on.<sup>117</sup> And have been explored for liquid detection, characterization, and biochemical differences in content. These include Raman spectroscopy, surface enhanced raman spectroscopy (SERS), surface plasmon resonance spectroscopy (SPR), and infrared spectroscopy (FTIR).<sup>117,118</sup> However, only some relevant research results in monitoring sEVs and imaging sEVs distribution exist. In this field, Boppart et al's<sup>119–122</sup> research team achieved certain achievements in sEVs monitor imaging using multi-harmonic generation (MHG) and multiphoton autofluorescence (MPF) (Figure 4). In 2018, the team conducted intraoperative imaging of human breast tissue using its customized portable nonlinear optical imaging system to process the third harmonic (THG) to generate sEVs distribution images using its custom segmentation algorithm. They confirmed the higher density distribution of sEVs in highly graded tumor tissue and suggested the potential of sEVs as a label-free biomarker for cancer diagnosis and prognosis.<sup>119</sup> Later, the team further investigated the label-free imaging of sEVs, which was accomplished by using multiphoton for direct visualization, characterization, and kinetic analysis of sEVs of breast cancer cells in different states based on their enriched NAD(P)H.<sup>120</sup> Since MPF can only image auto-fluorescent material, but most of the chemical components in sEVs are non-fluorescent, imaging with MPF may suffer from data loss. Thus, the team introduced coherent anti-Stokes Raman scattering (CARS) to provide a more detailed chemical composition analysis. The team performed MPF and CARS imaging of rat mammary glands and performed K-means clustering analysis of CARS under the visualization and guidance of MPF to obtain more chemical information to classify the seen sEVs. They screened a group of sEVs suspected to be associated with cancer, which was validated in tissue images of human mammary tumors.<sup>121</sup> The team also introduced fluorescence lifetime imaging microscopy (FLIM) for imaging sEVs, as FLIM can analyze the NAD(P)H status within sEVs and differentiate NADPH from NADP. It validated this experimentally and found that FLIM has the potential for imaging and characterizing sEVs to assess sEVs heterogeneity and to reflect cellular metabolic status by analyzing sEVs.<sup>122</sup>

## Summary and Outlook

All known methods of sEVs labeling have different advantages and disadvantages, the scope of application and limitations due to their different markers. Fluorescent dye labeling, as the most classical sEVs labeling technique, has the advantages of simple operation and low cost. However, based on the limitations of the current sEVs separation technology, it cannot effectively separate sEVs and small particles of lipids and proteins, while some lipophilic dyes will self-aggregate, which leads to unavoidable non-specific signals. At the same time, the influence of larger dye molecules on sEVs function, the instability of dye molecules, background fluorescence, and other drawbacks leave much room for optimization. Fluorescent dyes based on covalent binding for staining have increased specificity compared to common fluorescent dyes due to the relatively specific covalent binding reaction. Current click chemistry-based covalent binding labeling technology has dramatically improved the specificity and flexibility of labeling. Non-covalent conjugated labeling based on immunolabeling is another way to improve





**Figure 4** (A) Determination of phase of tumor cell invasion around desmoplasia by EVs distribution. (B) Intraoperative label-free multimodal imaging system. (A) and (B) Reprinted from Sun Y, You S, Tu H, et al. Intraoperative visualization of the tumor microenvironment and quantification of extracellular vesicles by label-free nonlinear imaging. *Sci Adv*. 2018. Creative Commons.<sup>119</sup> (C) In vivo visualization of EVs from cancer tissue in rat mammary tumors by label-free multiphoton microscopy. Reprinted from You S, Barkalifa R, Chaney EJ, et al. Label-free visualization and characterization of extracellular vesicles in breast cancer. *Proc Natl Acad Sci U S A*. 2019;116(48):24012–24018. Creative Commons.<sup>120</sup> (D) EVs cluster distribution in rat mammary tissues by K-means clustering of CARS spectra. Reprinted with permission from © The Optical Society. Sun Y, Chen EVV, Thomas J, Liu Y, Tu H, Boppart SA. K-means clustering of coherent Raman spectra from extracellular vesicles visualized by label-free multiphoton imaging. *Opt Lett*. 2020;45(13):3613–3616.<sup>121</sup>

the flexibility and sensitivity of labeling, and the technical requirements and experimental costs are relatively low. However, the large number of antibodies and reporter molecules bound to the surface of sEVs will inevitably affect the physical properties and physiological functions of sEVs. Therefore, non-covalent binding labeling techniques based on smaller molecules such as aptamers, aptamer ligands, anchoring peptides, and electrostatic interactions have also been developed and applied. However, the use of these techniques is still at an initial stage. Genetic engineering-based fluorescent protein and luciferase labeling technology are more efficient, specific, flexible, and safe. However, it is relatively difficult to construct cell lines that express the correct stable and efficient fusion proteins.

For simple sEVs labeling and tracking experiments, traditional fluorescent dyes may be the most cost-effective and convenient choice. However, it should be noted that the use of PKH dyes for sEVs labeling should be avoided for its adverse performance, and the R18 dyes for sEVs labeling could be used to observe sEVs membrane fusion. If



experimental cells are highly sensitive to dye toxicity or high imaging quality is required, attention can be directed towards newly developed novel dyes, some of which are commercially available. Immunolabeling techniques based on antigen–antibody reactions are another widely used fluorescence labeling method. They offer higher specificity compared to dye-based labeling methods, but staining efficiency is relatively lower. These techniques can be used for further subtyping and identification of sEVs based on choosing different antibody targets. However, immunolabeling should be avoided in experiments focusing on sEVs functionality due to its potential impact. If fluorescently labeled sEVs are required for long-term and large-scale experiments, biotechnological approaches may be a better choice. The characteristics and application of these fluorescent labeling techniques are summarized in [Table 1](#).

Labeling techniques based on radioisotope, nanoparticle, and chemical developers are more suitable for studying sEVs biodistribution. Their high sensitivity and resolution make them more suitable for deep tissue and in vivo tracer imaging. Thus, they have more greater clinical research value. However, the high research threshold of these labeling techniques, such as the use of isotopes, synthesis of nanoparticles, complex experimental operations, and the use of infrared imagers, photoacoustic imagers, MRI, and CT (even SPECT and PETCT), make it difficult to popularize these labeling techniques in some small and medium-sized laboratories. If qualifications and equipment allow, radioisotope labeling can achieve excellent imaging results and eliminate complex material synthesis steps. However, it requires the selection of suitable labeling media. For experiments involving sEVs-targeted therapy, AU nanoparticles with PTT effects and SPIONs, with excellent drug loading capabilities are excellent choices. Since different labeled imaging techniques have their own advantages and disadvantages, polymorphic imaging based on synthetic nanoparticles is a major research direction at present, and comprehensive analysis of information on the advantages of various imaging techniques can more effectively explore factual information on the processes of sEVs formation, release, circulation, uptake, and delivery of contents. All the aforementioned exogenous labeling methods have varying degrees of impact on sEVs. Therefore, label-free imaging techniques that have zero impact on sEVs hold great research prospects. These techniques, which require no labeling procedures, are extremely convenient and efficient. They enable in-situ detection of sEVs, leading to unparalleled advancements in clinical diagnostics. However, current research as a field is in its infancy, and the widespread adoption and dissemination of these technologies pose significant challenges. The characteristics and application of these non-fluorescent labeling techniques are summarized in [Table 2](#).

With the advancement of sEVs research, the stability of labeling techniques and their impact on the biological function of sEVs have gained increasing attention. The stability of sEVs labeling is crucial for maintaining their traceability and detectability. Key factors include selecting stable labeling agents, appropriate labeling methods, and optimization of labeling conditions. For fluorescence labeling, fluorescence quenching is a significant factor to consider. Therefore, choosing dyes with high photostability can enhance the stability of labeling. Additionally, direct conjugation of fluorescent dyes can lead to dye transfer, which affects signal interpretation and weakens the fluorescence signal. Thus, using covalent or non-covalent labeling methods can improve labeling stability. Radioisotopes with poor biocompatibility require suitable chelators for selective labeling of the sEVs membrane or its contents, because direct labeling with radioisotopes has low efficiency and poor stability. Metal nanoparticles have improved their biocompatibility through materials engineering approaches. They are usually internalized into sEVs through donor cell uptake for labeling purposes. Regarding the impact on exosome function, membrane labeling is a simple and efficient method. However, the membrane plays a crucial role in the physiological function of sEVs and excessive labeling modifications on the membrane, including using large dye molecules, hierarchical connections of antigen–antibody complexes, or chelation of particles on the membrane surface, can significantly affect their function. Therefore, surface labeling techniques based on smaller molecules and intraluminal labeling techniques may be preferable for functional studies. It is recommended to perform functional assessments after sEVs labeling to evaluate the potential impact of labeling on their functionality. This can be done through nanoparticle tracking analysis, cellular uptake experiments, protein analysis, or functional assays to assess the uptake capacity, membrane fusion ability, and biological activity of labeled sEVs.

As a hot research topic in recent years, sEVs research is undoubtedly promising, and labeling and tracer imaging techniques provide the necessary technical support for sEVs-related studies in vivo and in vitro. However, various labeling techniques have their own advantages and disadvantages, and the appropriate labeling strategy should be chosen

**Table 1** Fluorescent Labeling Techniques of sEVs Imaging

Technique	Agent	Wavelength ( $\lambda_{ex}/\lambda_{em}$ )	Emitted Light	Modality	Target	Time	Feature	Ref
Membrane fluorescent dye	Dil	551/570 nm	Orange	FM	Lipid (M)	30min	-	[6]
	DiO	483/501 nm	Green	FM, FCM	Lipid (M)	30min	-	[7]
	DiD	646/663 nm	Red	FMT	Lipid (M)	30min	In vitro	[10]
	DiR	710/780 nm	NIR	FM	Lipid (M)	30min		[9]
	FM 4-64	558/734nm	Red	FM	Lipid (M)	15min	Low cross-staining	[11]
	PKH26	551/567nm	Orange	CLSM, RIM	Lipid (M)	10min, 4min	Serious accumulation	[13,15]
	PKH67	490/502nm	Green	CLSM	Lipid (M)	10min, -	Size impact	[13,14]
	Mem	494-652 nm/512-672nm	Green to Red	CLSM	Lipid (M)	30min	No accumulation	[18]
							Low size influence	
	CellMask™	522-649 nm/535-666nm	Green to Red	FM	Lipid (M)	70min	Slow internalization	[19]
							Cannot permeabilization	
	MemBright™	499-689/506-713nm	Green to Red	CLSM, FM	Lipid (M)	30min	Low concentration	[21,22]
							Self-quenching	
	3-NBD-cholesterol	470/541nm	Green	FCM	Lipid (M)	48h	For studies of cholesterol transfer	[24]
Bio-Conjugation	WGA-647	650/670nm	Red	SMLM	Glycoprotein (M)	30min	Surviving from permeabilization	[26]
	R18	560/590nm	Orange	FM	Lipid (M)	30min	Membrane fusion studies	[27]
	CFDA-SE	500nm/520nm	Green	FMi-OOC	Amino (I)	30min	Inadequacy for acid	[35]
	ExoTracker	550nm/670nm	Red	CLSM	Amino (M)	-	Suitable for acid	[37]
	TAMRA-NHS	553nm/575nm	Orange	FM	Peptide (M)	1h	-	[38]
	C5-maleimide-AF488	494nm/517nm	Green	CLSM	Tetraspanin (M)	5min	Specificity	[40]
							No influence	
	DBCO-NHS+AF488-azide	480nm/520nm	Green	CLSM	Amino (M)	1h+4h	Flexibility	[42]
	AHA/ManNAz+DBCO-Cy3	550nm/570nm	Orange	FCM, CLSM	-(M)	4h	Glycan analysis	[43]
Non-covalent	CD63/HER2-AF647	642nm/670 nm	Red	PALM/STORM	-	2h+1h	Specificity	[46]
							Functional influence	
	TSG101/CD63/calnexin-AF594	590nm/620 nm	Red	CLSM	-	1.5h+1h		[47]
	LZH8-NA-FITC	490 nm/520 nm	Green	FCM	HepG2 (M)	30min	Specificity	[48]
	MJ5C	-	Green	CLSM	PD-L1 (M)	40min	Biocompatibility	[51]
	Rhodamine-CP05	525 nm/625 nm	Red	FM, FCM,	CD63(M)	12h	Specific binding to CD63.	[49]
	AF680-CP05	680 nm/705 nm	Red	IVIS				
	DPA-SCP	488nm/500-700 nm	Red	CLSM, IVIS	Charge(M)	2h	In vitro	[55,56]
							Electrostatic adsorption	

(Continued)

**Table I** (Continued).

Technique	Agent	Wavelength ( $\lambda_{ex}/\lambda_{em}$ )	Emitted Light	Modality	Target	Time	Feature	Ref
Cell-Engineering          QD	CD63-GFP	488 nm/507 nm	Green	CLSM	-	-	Stable labeling	[58]
	CD63-iRFP682	663 nm/682 nm	Red	SIM	-	-	High technical requirements	[59]
	palmNG	507 nm/514 nm	Green	CLSM	-	-	Limited clinical application	[60]
	PalmGFP	488 nm/507 nm	Green	CLSM,	-	-		[61]
	PalmtdTomato	554 nm/581 nm	Red	MP-IVM				
	gLuc-LA	$\lambda_{em}=480nm$	Blue	IVIS	-	-		[64]
	Rluc	$\lambda_{em}=460nm$	Blue	CLSM, IVIS	-	-		[65]
	CD63NanoLuc	$\lambda_{em}=420nm$	Blue	IVIS	-	-		[66]
	InP/ZnS QD	596/615 nm	Red	FM, SPR		4h	SPR imaging; Multiplexing	[102]
	Si QD	405/420-490 nm	Green	SMLM	CD63(M)	8h	SMLM imaging; Biocompatibility	[103]
	GCDs	405/495–575 nm	Red	FM	HER2(M)	3h	Biocompatibility	[104]
	QD605-EpCAM	$\lambda_{em}=605nm$	Red	CLSM	EpCAM(M)	-	Quantify sEVs abundance	[105]
	QD655-EphA2	$\lambda_{em}=655nm$			EphA2(M)		For clinical analysis	
	4FB-HyNic-QD	$\lambda_{em}=585nm$	Red	CLSM	Amino(M)	2h+2h	Very stable High resolution live imaging ability	[106]

**Abbreviations:** CLSM, Confocal laser scanning microscope; FCM, Flow CytoMetry; FM, Fluorescence microscope; FMi-OOC, Fetal–Maternal interface Organ-on-chip; I, Inside; IVIS, In vivo optical imaging; M, Membrane; RIM, Retinal imaging microscopy; SIM, Structured Illumination Microscopy; SMLM, Single molecule localization microscopy; SPR, surface plasmon resonance; STORM, Stochastic optical reconstruction microscopy.

**Table 2** Non-Fluorescent Labeling Techniques of sEVs Imaging

Technique	Agent	Injection	Modality	Target/Method	Time	Application	Ref
Radiolabeling	<sup>131</sup> I	IV	SPECT/CT	Iodination(M)	30min	Biodistribution	[70]
	<sup>125</sup> I-IBB	IV	γ-Counter	SAV-LA(M)	30min	Pharmacokinetics	[71]
	[ <sup>124</sup> I]NaI	IV	PET, γ-Counter	Iodination(M)	2h	Biodistribution	[72]
	[ <sup>111</sup> In] Trop	IV	SPECT/CT, γ-Counter	Shuttling (I)	20min	Biodistribution	[73]
	[ <sup>111</sup> In]DTPA			Chelation (M)			
	<sup>99m</sup> TcCl <sub>4</sub>	IV, IP, IN	SPECT/CT	Adsorption (M)	30min	Biodistribution Pharmacokinetics	[74]
	<sup>99m</sup> Tc-HMPAO	IV	SPECT/CT	Glutathione (I)	1h	Biodistribution	[75]
	( <sup>99m</sup> ) Tc-tricarbonyl	IV	SPECT/CT	Chelation (M)	30min	Biodistribution	[76]
	[ <sup>99m</sup> Tc (CO) <sub>3</sub> (H <sub>2</sub> O) <sub>3</sub> ] <sup>+</sup>	IV	γ-Camera	DARPin G3(M)	1h	Biodistribution	[77]
	[ <sup>89</sup> Zr] Zr(oxinate) <sub>4</sub>	IV	PET, γ-Counter	Lysine residues(M)	1h	Biodistribution Pharmacokinetics	[78]
	[ <sup>89</sup> Zr] Zr(oxinate) <sub>4</sub>	IV	PET-CT	Shuttling (I)	20min	Biodistribution	[79]
	SCN-NOTA <sup>64</sup> Cu/ <sup>68</sup> Ga	IV, SC	PET, γ-Counter	Chelation (M)	5min,30min	Biodistribution	[80]
	<sup>64</sup> Cu-CB-TE1AIP	IV	PET	Chelation (M)	3h	Pharmacokinetics	[81]
	Deuterium	-	SERS	Metabolism(W)	-	Tracking	[82]
Nanoparticles	Glu-GNPs	IV, IN	CT	Cell Internalization (I)	3h	Biodistribution	[83–85]
	AuNP-PEG-FA	IV	FM, SPECT/CT	Folic acid receptor(I)	2–24h	Biodistribution	[86]
	Au Nanostars	IV	PAI	Cell Internalization(I)	2d	Biodistribution, Therapy	[88]
	USPIO	IM	MRI	Cell Internalization(I)	1d,3d	Biodistribution	[92,93]
	SPIONs	IV	MRI	Electroporation(I)	1ms	Biodistribution, Therapy	[94]
	SPIONs	IV	MRI	Electroporation(I)	1ms	Biodistribution	[95]
	GdL	IV	MRI	Lipid insertion(M)	30min	Biodistribution	[98]
	Ag <sub>2</sub> Se@Mn QDs	IP	IVIS, MRI	Electroporation(I)	30min	Biodistribution	[107]
	GION	IP	MRI (PAI, CT, SERS)	Lipid insertion(I)	-	Biodistribution, Therapy	[108]
	NCs (λ <sub>ex</sub> /λ <sub>em</sub> = 500/750 nm)	IV	FM, MRI, CT	Biosynthesis(I)	-	Biodistribution, Diagnosis	[109]
	V2C-QDs (λ <sub>ex</sub> /λ <sub>em</sub> = 405/469nm)	IV	CLSM, PAI, MRI	RGD(I)	-	Biodistribution, Therapy	[110]
	Ag-Fe <sub>3</sub> O <sub>4</sub> NCs	-	CLSM (CT, MRI)	Biosynthesis(I)	-	Uptake	[111]
	Ce6	IV	PAI	Re-assemble(M)	1h	Biodistribution, Therapy	[113]
	FTHI	IM	MRI	Biosynthesis(I)	-	-	[115,116]
Chemical contrast agent							
Label-free	-	-	Multimodal imaging system (MHG +MPF)	-	-	Intraoperative imaging	[119]
	NAD(P)H	-	Multiphoton Microscopy (MPF)	NAD(P)H	-	Dynamics	[120]
	-	-	Multimodal imaging system (MPF+CARS)	-	-	Biodistribution	[121]
	NAD(P)H	-	FLIM	NAD(P)H	-	Characterizing	[122]

**Abbreviations:** CARS, Coherent anti-Stokes Raman scattering; CLSM, Confocal laser scanning microscope; CT, Computed Tomography; FLIM, Fluorescence lifetime imaging microscopy; FM, Fluorescence microscope; I, Inside; IM, Intramuscular; IN, Intranasal; IV, Intravenous; IVIS, In vivo optical imaging; IP, Intraperitoneal; M, Membrane; MHG, Multiharmonic generation; MPF, Multiphoton autofluorescence; MRI, Magnetic Resonance imaging; PET, Positron emission tomography; SC, Subcutaneous; SERS, Surface enhanced Raman spectroscopy; SPECT, Single photon emission computed tomography SPR, Surface plasmon resonance; W, The whole sEVs.

according to the experimental purpose. The development of marker imaging technology also advances our understanding of sEVs and makes them ultimately useful for clinical purposes.

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## Disclosure

The authors report no potential conflicts of interest in this work.

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