Received: 23 July 2019 | Revised: 3 September 2019 | Accepted: 4 September 2019

DOI: 10.1002/wnan.1594

ADVANCED REVIEW

WIREs NANOMEDICINE AND NANOBIOTECHNOLOGY WILEY

Advances in imaging strategies for in vivo tracking of exosomes

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Funding information
Israel Science Foundation ISF, Grant/Award Number: 749/14; National Natural Science Foundation of China, Grant/Award Number: 51761145041; Ministry of Science, Technology and Space Israel, Grant/Award Number: 3-15677; Ministry of Science and Technology Israel-China; Israel Science Foundation Joint NSFC-ISF Research Grant, Grant/Award Number: 2533/17

Abstract

Exosomes have many biological functions as short- and long distance nanocarriers for cell-to-cell communication. They allow the exchange of complex information between cells, and thereby modulate various processes such as homeostasis, immune response and angiogenesis, in both physiological and pathological conditions. In addition, due to their unique abilities of migration, targeting, and selective internalization into specific cells, they are promising delivery vectors. As such, they provide a potentially new field in diagnostics and treatment, and may serve as an alternative to cell-based therapeutic approaches. However, a major drawback for translating exosome treatment to the clinic is that current understanding of these endogenous vesicles is insufficient, especially in regards to their in vivo behavior. Tracking exosomes in vivo can provide important knowledge regarding their bio-distribution, migration abilities, toxicity, biological role, communication capabilities, and mechanism of action. Therefore, the development of efficient, sensitive and biocompatible exosome labeling and imaging techniques is highly desired. Recent studies have developed different methods for exosome labeling and imaging, which have allowed for in vivo investigation of their bio-distribution, physiological functions, migration, and targeting mechanisms. These improved imaging capabilities are expected to greatly advance exosome-based nanomedicine applications.

This article is categorized under:
Therapeutic Approaches and Drug Discovery > Emerging Technologies
Diagnostic Tools > In Vivo Nanodiagnostics and Imaging
Nanotechnology Approaches to Biology > Nanoscale Systems in Biology

KEYWORDS
exosomes, in vivo imaging, nanoparticles, tracking

1 | INTRODUCTION

1.1 | Exosomes biogenesis

Extracellular vesicles (EVs) are cell-derived vesicles with proteins and nucleic acids that are packaged in a phospholipid-bilayer membrane. There are three main types of EVs: exosomes, microvesicles, and apoptotic bodies, these types differ by their biogenesis, size, and membrane composition. Exosomes, a subtype of EVs, were originally considered to be known as

janitors, disposing unwanted materials from cells; however, more recent in-depth research has shown their actual biological functions as nanocarriers for cell-to-cell communication that allow the exchange of complex information between cells, affecting homeostasis maintenance, modulation of the immune response, and angiogenesis, in both physiological and pathological conditions (Bunggulawa et al., 2018; Vader, Mol, Pasterkamp, & Schiffelers, 2016; B. Yu, Zhang, & Li, 2014). According to the new guideline MISEV2018 from the International Society for Extracellular Vesicles (ISEV), exosomes should be classified according to physical characteristics of the extracellular vesicles such as size (Théry et al., 2018). Herein, we use the general term "exosomes" to specify small EVs (sEVs) with size smaller than 200 nm. The contents of exosomes and their physiological functions are affected mostly by their cell of origin type and physiological state. Exosomes have a complex composition of proteins, nucleic acids, lipids, and metabolites (Ha, Yang, & Nadithe, 2016; Jiang & Gao, 2017; Johnsen et al., 2014; Kumar, Michael, Park, Granick, & Cho, 2018).Exosomes interact with recipient cells via three main pathways: by direct fusion to the cell membrane, via endocytic uptake, or they may connect to the cell surface by a lipid–ligand receptor interaction and transmit content (Keshtkar, Azarpira, & Ghahremani, 2018).

1.2 | Exsosome functions

Recent studies have revealed the clinical significance of exosomes in disease diagnosis, drug-delivery, and therapy. Exosomes which contain proteins, mRNA, or miRNA, can transport this information content to neighboring or distant cells and have influence over the microenvironment and the recipient cells (Bier et al., 2018; Ha et al., 2016; Jiang & Gao, 2017; Johnsen et al., 2014; Kumar et al., 2018; R. C. Lai, Yeo, Tan, & Lim, 2013; Lu et al., 2017; Luarte, 2016; Munson & Shukla, 2015).

Exosome-based vehicles may have multiple advantages over currently available drug delivery vehicles: they are nontoxic and nonimmunogenic, they have the ability to overcome natural barriers like the blood–brain-barrier, they show stability within the blood circulation, and they can be engineered to have robust delivery capacity and targeting specificity (Tan, Rajadas, & Seifalian, 2013; van den Boorn, Schlee, Coch, & Hartmann, 2011). Due to the above advantages, and since promising results have been achieved in vitro and in animal models, several clinical studies are currently being performed (Rayyan, Zheutlin, & Byrd, 2018). Many of these studies are in the oncology field for melanoma, lung and colon cancer therapy using immune cell-derived exosomes (Fuster-Matanzo, Gessler, Leonardi, Iraci, & Pluchino, 2015), others are for neurological disorders, such as Parkinson disease (Dubal & Pleasure, 2019). Several have already reported important achievements (Besse et al., 2016; Wang, Zheng, & Zhao, 2017).

However, even in these studies there are plenty of mysteries surrounding the manner in which the exosomes achieve their desired task. The process from injection of the exosomes to the therapeutic effect is a black box, and many parameters are unknown yet. What is their mechanism of action? What does their biodistribution look like? What target do the exosomes reach? What targets are they not able to reach? Do exosomes behave differently when originated from different cells? In order to be able to answer these and similar questions, it is vital to develop methods of imaging and tracking the exosomes in vivo.

1.3 | Labeling exosomes

Tracking exosomes in living organisms is challenging. This is due to the exosomes' small size, their rapid dispersion in body fluids, and their composition, which is similar to that of body cells and therefore lacks contrast for imaging techniques (Hyenne, Lefebvre, & Goetz, 2017). Hence, to track exosomes in vivo, different strategies have been developed to label the exosomes. Most involve labeling them outside the organism, and then delivering them back into the body. Upon delivery, different imaging methods and modalities have been applied in order to visualize the labeled exosomes. Each technique has different capabilities and advantages (Di Rocco, Baldari, & Toietta, 2016).

Exosomes have a unilamellar lipid bilayer membrane which serves as a natural barrier to protect the exosome cargo from degradation in the bloodstream. Yet, this membrane as well as the endogenous content of the exosome make the loading of exosomes with drugs or contrast agents very challenging. Two main approaches exist for labeling exosomes: direct and indirect labeling pathways (Armstrong, Holme, & Stevens, 2017). Direct labeling is based on loading exosomes with labeling agents after their isolation, while indirect labeling involves manipulation of their parent cells by introducing exogenous agents that are then subsequently incorporated into the secreted exosomes. Both pathways are suitable and reliable for real time in vivo imaging of exosomes (Gangadaran, Hong, & Ahn, 2017).

Another important factor that affects the labeling procedure is the type of the labeling, which can be either endogenous or exogenous. The endogenous labeling technique hijacks cell biosynthesis to favor production of specific endogenous fluorescent markers or fluorescent proteins, which then become part of the exosome membrane. This type of labeling marks the

exosome conclusively so that tracking these agents definitely implies tracking of the exosomes (Hoffman, 2017). The exogenous labeling technique uses an external agent that resides within the exosome, such as nanoparticles and fluorescent dyes. This type of labeling is easier to apply, and allow the use of additional in vivo imaging modalities such as magnetic resonance imaging (MRI) and computed tomography (CT). However, these labeling agents can potentially diffuse out of the exosomes (e.g., when the exosome raptures) and therefore careful verification and interpretation of the results is needed (Meir & Popovtzer, 2018).

The aforementioned advantages and limitations should be taken into consideration when choosing one labeling method over another. An ideal labeling method should be safe for the exosome, specific and sensitive for the imaging modality, easy to apply, and with high signal-to-noise ratio.

1.4 | In vivo imaging methods for exosome tracking

Translating exosome therapy to the clinic is challenging. Currently, assessing the success or failure of treatment can only be done by evaluating symptom improvement usually weeks or months after treatment. To address this challenge, the fate of the administered exosomes should be assessed in real time. This requires a reliable, noninvasive, exosome in vivo imaging technique, which can provide insight on the functionality, viability and trafficking of these vesicles within the body. The current available imaging technologies include optical, tomographical, and nuclear imaging modalities, in which each one of them has different capabilities related to biocompatibility, penetration depth, spatial and temporal resolution, half life-time, and clearance rates.

Understanding the available imaging techniques for exosome tracking is essential for selecting the most appropriate imaging technology to achieve the desired imaging and therapeutic goals (Shaikh et al., 2018). Table 1 summarizes the current main methods detailed in this review. (Table 1).

1.4.1 | Optical imaging

Optical imaging is a widespread technique in molecular and cellular biology with the advantage of high-throughput efficiency at low cost. It consists of two main types: bioluminescent imaging (BLI) and fluorescence imaging (FLI). Fluorescent-probe-labeled analytes emit a fluorescence signal upon excitation, which is recorded by the imaging modality. Fluorescence microscopy is easy to operate, enables to trace analytes in real time and noninvasively, and uses nonionizing light-sources. As discussed previously, it can also be relatively simple to label exosomes fluorescently. FLI can monitor the behavior of a single cell in real time, which is beneficial for observing the interactions between exosomes and cells (Boddington, Henning, Sutton, & Daldrup-Link, 2008). Although there is a growing variety of fluorescent probes for exosome imaging, there are several limitations for in vivo tracking using FLI technique such as poor penetration depth and spatial resolution which makes it unsuitable for clinical translation, lipophilic dyes promote exosome aggregation, and the presence of auto-fluorescence artifacts (since the emission peak of certain dyes can overlap with the fluorescence emission of biological tissues) (Ntziachristos, Bremer, & Weissleder, 2003).

Tracking of exosomes using FLI

FLI involves using endogenous or exogenous molecules or materials which emit light upon activation by an external light source. Fluorescent proteins (such as GFP and RFP) are mostly introduced to the parent cells—indirect labeling, while lipophilic dyes (such as Cy7, PKH26, luminogens) are incorporated into the exosomes directly. Labeling exosomes with lipophilic dyes provides insights mainly regarding their biodistribution and organotropic uptake while labeling exosomes with fluorescent proteins, can shed light on the biological mechanisms that occur in the cell and involve exosome biogenesis, secretion, and communication (Hoffman, 2017). FLI is considered a straightforward, efficient, and relatively low-cost labeling and imaging technique (Hoffman, 2017; Thomas, 2015).

Smyth et al. used exosomes derived from cancer cells (4T1, PC3 and MCF-7) that were labeled with lipophilic carbocyanine DiOC18(7) (DiR) and imaged with combined two-dimensional (2D) optical and three-dimensional (3D) optical tomography after injection to 4T1 tumor-bearing mice. They examined their biodistribution compared to liposomes, and assessed their doxorubicin delivery efficacy. in vivo imaging revealed that the majority of intravenously (iv) injected exosomes accumulated in the liver and spleen 1 hr postadministration with no significant changes up to 24 h, and displayed rapid clearance and low accumulation in the tumor. Intratumorally (IT) injected exosomes showed higher retention than liposomes within tumor tissue, and doxorubicin-loaded exosomes showed higher efficacy when compared to liposomes

TABLE 1 current main methods for labeling and in vivo imaging of exosomes

Imaging technique	Imaging modality	Labeling agent	Labeling method	Exosome source cells	Imaging ROI	Imaging time points	Admin route	References
Optical imaging	FLI	DiR	Direct-incubation	Breast cancer cells (4T1, PC3, MCF-7)	Whole body	1, 8, 24 hr 20 min, 2 hr	iv and IT	Smyth et al. (2015)
		Cy7	Direct-incubation	4T1 cancer cells	Whole body	After injection	iv	(Jung, Youn, Lee, Kang, and Chung (2017)
		DiD	Direct-incubation	Mesenchymal stem cells	Whole body	15 min, 5, 24 hr	iv	Grange et al. (2014)
		DPA-SCP luminogen	Direct-incubation	Mesenchymal stem cells	Whole body	1 hr, 3, 5, 7, 12 days	iv	Cao et al. (2019)
		DBCO-Cy7	Direct-incubation	Breast cancer cell lines (MCF-7, MDAMB-231, HS578T)	Whole body	24 hr	iv	Zhang et al. (2018)
		CFP (and Cre)	Indirect	Cancer cells (T47D, MDA-MB-231)	Tumor ROI	—	Endogenous exosomes	Zomer et al. (2015)
		PalmGFP and PalmtdTomato	Indirect	EL4 lymphoma tumor cells	Tumor ROI	9 days	IT	Lai et al. (2015)
		CD63-pHluorin	Indirect	Endogenous exosomes	Whole fish	3 days	Endogenous exosomes	Verweij et al. (2019)
		ADIBO-Cy3 and ADIBO-Cy5.5	Direct-incubation	Cancer cells (MDA-MB-231, MCF7)	Excised organs	1 day	iv	Lee, Kim, Zhang, Song, and Tung (2018)
	BLI	Gluc and lactadherin	Indirect	Murine melanoma (B16-BL6)	Whole body	10, 30, 60, 240 min	iv	Takahashi et al. (2013)
		Gluc and Biotin acceptor peptide	Indirect	Human embryonic kidney cells (HEK-293T)	Whole body	30 min	iv	C. P. Lai, Mardini, et al. (2014), C. P. Lai, Tannous, and Breakefi (2014a), and C. P. Lai, Tannous, and Breakefield (2014b)
		Fluc, mCherry, and palmtdTomato	Indirect	Human glioblastoma (GBM)	Brain ROI	Every 5 min for 30 min	Endogenous exosomes	van der Vos et al. (2016)
		Rluc	Indirect	Anaplastic thyroid cancer cells (CAL62)	Whole body	5, 30, 60 and 120 min	iv	Gangadaran et al. (2018)
Nuclear imaging	SPECT/CT	^{111}In-oxine	Direct-incubation	Cancer cells (4T1, PC3, MCF-7)	Whole body	24 hr	iv	Smyth et al. (2015)

(Continues)

TABLE 1 (Continued)

Imaging technique	Imaging modality	Labeling agent	Labeling method	Exosome source cells	Imaging ROI	Imaging time points	Admin route	References
		^{99m}Tc-HMPAO	Direct-incubation	Macrophage cells (mouse raw 264.7)	Whole body	0.5, 3, 5 hr	iv	Hwang et al. (2015)
		^{99m}TC-tricarbonyl	Direct-incubation	Erythrocyte	Whole body	1 hr	iv	Varga et al. (2016)
		^{111}Indium	Direct-incubation	Melanoma (B16-BL6)	Whole body	0, 4, 24 hr	iv	Faruqu et al. (2019)
	Gamma camera	^{99m}Tc	Direct-incubation	Exosome-mimetics derived from red blood cells	Whole body	1, 3 hr	iv	Gangadaran et al. (2018)
Tomography	CT	GNP	Direct-incubation	Mesenchymal stem cells	Whole brain	1, 3, 24 hr	IN	Betzer, Meir, et al. (2017), Betzer, Perets, et al. (2017) and Betzer, Shilo, et al. (2017)
		GNP	Direct-incubation	Mesenchymal stem cells	Whole brain	1, 3, 24, 96 hr	iv and IN	Perets et al. (2019)
	MRI	SPION	Direct-electroporation	B16-F10 melanoma cells	Lymph nodes ROI	1, 48 hr	iv	Hu, Wickline, and Hood (2015)
		USPIO	Indirect	Adipose stem cells	Muscle ROI	After injection	im	Busato et al. (2016)
		GION	Direct-extrusion	Tumor cell lines	ROI tumor	12 days	iv	Jc Bose et al. (2018)
	MPI (+CT)	SPIO	Indirect	MDA-MB-231 human breast cancer cells	Injection ROI	After injection	IT	Jung, Jo, Yu, Gambhir, and Pratx (2018)
Photoacoustic	PAI	SPION	Indirect	Triple-negative breast cancer cells	ROI tumor	4, 24 hr	iv	Piao et al. (2018)
		Graphene quantum dot nanozyme	Incorporated during nanozyme preparation	Red blood cells engineered exosomes	Tumor ROI	2, 4, 8 hr	iv	Ding et al. (2019)

Abbreviations: BLI, bioluminescent imaging; CT, computed tomography; FLI, fluorescence imaging; GION, gold–iron 70 nm nanoparticles; GNP, gold nanoparticles; im, intramuscular; iv, intravenous; MPI, magnetic particle imaging; ROI, regions of interest; SPECT, single-photon emission CT; SPION, superparamagnetic iron oxide nanoparticles.

(Smyth et al., 2015). MicroRNA (miR) is reported to have a significant role in tumor growth and angiogenesis. Jung et al. studied if and how hypoxic 4T1 cancer cells exosomes mediate transfer of miR-210 to neighboring cells, and their effect on the tumor microenvironment. Exosomes that were directly labeled with Cy7 administered iv and imaged using IVIS spectrum showed a tendency to accumulate mainly in the tumor tissue and intestine. Analysis of mouse serum also indicated systemic circulation of exosomes containing miR-210 (Jung et al., 2017). Exosomes derived from mesenchymal stem cells (MSCs) have the potential for therapy of injured tissue, as they preserve some of the therapeutic characteristics and factors of their parent cells (R. C. Lai et al., 2013). To compare the efficacy of direct and indirect labeling methods for in vivo detection of exosomes at the site of damage, mice with glycerol-induced acute kidney injury were injected with MSCs-derived exosomes that were either directly stained with a near infrared lipophilic cyanine dye, DiIC18(5) (DiD), or indirectly stained by preincubating MSCs with the dye and collecting the exosomes. Exosomes injected iv were monitored using combined 2D and 3D optical tomography 15 min, 5 and 24 hr postinjection. Both labeling techniques were found to be suitable for in vivo detection of the localization of exosomes mainly within the kidneys after 5 hr. However, Indirect labeling showed lower fluorescence intensity at all time points (GRANGE et al., 2014). MSC-derived exosomes were labeled with the aggregation-induced emission luminogen—DPA-SCP and tracked in a mouse acute liver injury model. Luminogens are dyes that become strong emitters when aggregated, thus overcoming fluorescent quenching caused by aggregation of fluorescent molecules. MSC-derived exosomes injected iv to mice with acute liver injury were imaged using IVIS, 1 hr, 1, 3, 5 and 7 days postinjection, and to wild type mouse up to 12 days postinjection. Fluorescent signals were detected as soon as 1 hr postinjection and peeked after 1 day in the liver, then gradually decreased but were still detected in the liver after 6 days. The labeling efficiency of DPA-SCP was found to be superior to those of other popular trackers such as PKH26 and DiI. However, the fluorescence intensity of DPA-SCP when injected without exosomes to wild type mice was also very high for the entire experiment duration, which makes it hard to distinguish between the groups (Cao et al., 2019). In order to avoid altering exosome structure and composition, Zhang et al. aimed to label the phospholipid by biorthogonal chemistry (without interfering with native biochemical processes of the cell). They synthesized a choline analogue with azide group on the outer membrane of the secreted exosomes that have the ability to conjugate several dyes (such as Cy7, Cy5, and MB 594). Exosomes originating from different cells exhibit different biodistribution and organotropism. Therefore, azide-labeled exosomes (20 μg) isolated from different human breast cancer cell lines (MCF-7, MDAMB-231, and HS578T), were injected iv to mice, and imaged using Maestro 24 hr postinjection. MDAMB-231 and HS578T derived exosomes show similar biodistribution, with fluorescence signals found in the brain (more than 10%ID), liver (more than 20%ID), kidney and lungs. MCF-7 derived exosomes tend to accumulate mainly in the liver and kidney (Figure 1). Measurement of the fluorescence intensity of Cy7 in the serum of exosome-injected mice 6 hr postinjection showed that the exosome level decreased over time, to 1% of the initial amount indicating fast blood clearance (Zhang et al., 2018). Exosome transfer between MDA-MB-231 and T47D breast cancer tumor cells was examined in vivo via combined high-resolution intravital imaging with the well-established Cre-LoxP recombinase technology (Ntziachristos et al., 2003). Exosomes were indirectly labeled via parent cells to express cyan fluorescent protein (CFP) and Cre recombinase induced a color switch from DsRed to eGFP, specifically in recipient cells. Recipient cells that did not take up exosomes were marked with DsRed (i.e., unrecombined reporter) and served as an internal control for eGFP-expressing recipient cells that had taken up exosomes. Importantly, the detection was restricted to uptake of exosomes released by Cre + cells. This technique enables the analysis of biological effects induced by exosomes released from an a priori defined tumor cell *population* in vivo. The study demonstrated live imaging of single exosome transfer between cells and the transfer of exosomes between tumor cells to healthy cells. The study also found that less malignant T47D tumor cells take up highly metastatic MDA-MB-231 derived exosomes when injected both as a mixture in one mammary gland or separately in contralateral mammary glands but with lower efficiency. Moreover, exosomes from more malignant MDA-MB-231 cells lead to increased metastatic potential of less malignant T47D Cells (Zomer et al., 2015). Another study used EL4 lymphoma tumor cells expressing GFP fused to a consensus palmitoylation (palmGFP) reporter sequence, for indirect labeling of exosomes. This reporter enabled use of multiphoton intravital microscopy for in vivo visualization and tracking of uptake and exchange of tumor-derived exosomes between cell populations. The exosomes in palmGFP-EL4 tumor-bearing mice were found either intracellular, or outside of EL4-PalmGFP tumor cells. The highest intensity of exosome secretion was observed in the tumor periphery while the tumor center exhibited low levels of exosomes. Time lapse imaging revealed that large exosomes (1 μm diameter) remain in close proximity to the tumor tissue for longer periods than smaller ones. Based on the imaging results, the authors suggested that the exosomes are attached to or are internalized by a motile, nonvisualized cell population, such as tumor-infiltrating immune cells (C. P. Lai, Mardini, et al., 2014). The zebrafish model, being a transparent vertebrate, allows in vivo imaging of whole animals, with fast embryonic development and high similarity to human organogenesis and physiology. Verweij et al. developed an integrated model to study exosome release, transfer, and function using zebrafish that

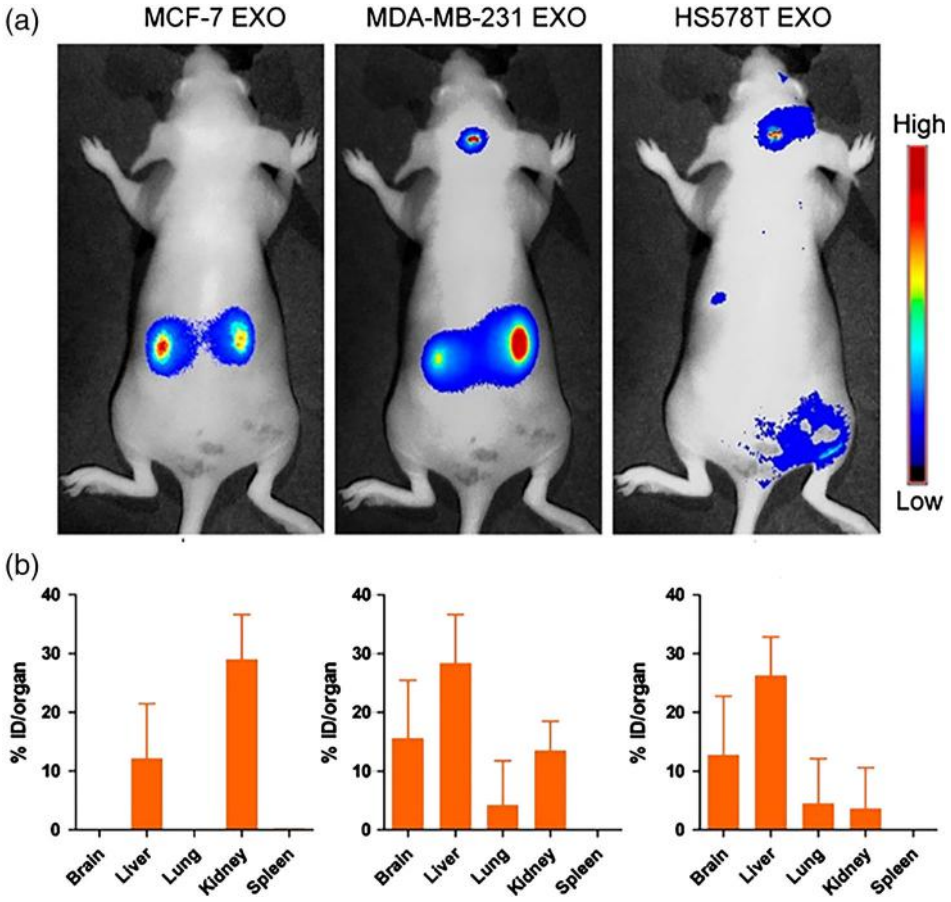

FIGURE 1 Kinetics of breast-cancer-cell-derived exosomes 24 hr postexosomes injection. (a) Mice following intravenous injections of fluorescently labeled MCF-7-, MDA-MB-231-, and HS578T-derived exosomes. (b) Biodistribution of exosomes, analyzed and quantified by recoding the photons/second/steradian (ph/s/sr) of each organ, normalized to the injected dose fluorescence intensity. The results are expressed as the means ± *SD* ($n = 3$) (Reprinted with permission from Zhang et al. (2018). Copyright 2018 American Chemical Society)

transiently expressed exosomes CD63 fused to pHluorin, a pH-sensitive GFP that emits light when exposed to neutral extracellular fluid. in vivo imaging by spinning disk confocal imaging 3 days postfertilization demonstrated release of CD63-pHluorin exosomes from the yolk syncytial layer into the blood flow. Labeled exosomes were found to be taken up by macrophages and endothelial cells of the caudal vein plexus (Verweij et al., 2019). Lee et al. suggested a metabolic exosome labeling technique, using natural metabolic incorporation of azido-sugar into the glycan, and copper-catalyzed azide-alkyne click reaction. Cells were loaded with tetra-acetylated *N*-azidoa-cetyl-D-mannosamine incorporated into glycans, the azido-containing exosomes were then collected and labeled with fluorescent dyes through click reaction. in vivo tracking of fluorescent labeled cancer-derived exosomes were evaluated in various cells (MCF7 and MDA-MB-231 cells) and tumor bearing mice. Highly metastatic cancer-derived exosomes homed to the tumor and showed selective organ distribution in vivo (mostly in the liver and intestines) (Lee et al., 2018).

Taken together, although FLI has drawbacks such as low penetration depth, labeling exosomes with fluorescent indicators provides information not merely about biodistribution and migration abilities of the exosomes, but also on the biological mechanisms that occur in the cell and involve exosomes biogenesis, secretion and communication with high resolution.

Tracking of exosomes using bioluminescence imaging

Another widely-used imaging method for studying biological activity is bioluminescence imaging (BLI), which uses light generated from a natural biological process, that is, the luciferase enzyme-substrate reaction, and an ultrasensitive camera for signal detection. Bioluminescence does not require an excitation source to emit light, but rather emits bioluminescence through reaction of the respective substrate with either ATP and Mg^{2+} or oxygen alone. BLI's high sensitivity and low background signal qualities make luciferase a good reporter for in vivo studies (Kim, Kalimuthu, & Ahn, 2015). For labeling exosomes with BLI components, the indirect labeling method is used. First, reporter genes such as *Gaussia princeps* luciferase (Gluc), firefly luciferase (Fluc), or Renilla luciferase (Rluc) are transduced into the parent cell line. The exosomes produced from the parent cells expressing the reporter protein will then carry the reporter protein inside their lumen or upon their membrane. For visualizing the exosomes through real time FLI, a substrate such as Coelenterazine needs to be injected (Badr & Tannous, 2011).

Studies show that labeling of exosomes using bioluminescence reporters is safe for the exosomes, the bioluminescence protein is retained in the exosomes even after iv injection, and the method can be used for exosome bio-distribution studies (C. P. Lai, Tannous, & Breakefi, 2014a; Takahashi et al., 2013).

Takahashi et al. were the first to reveal that exosomes can be visualized in vivo using bioluminescent reporter proteins. They used Gluc together with lactadherin, a membrane-associated protein found mainly in exosomes, to track murine melanoma B16-BL6 cell-derived exosomes after iv injection in mice and analyze the overall bio-distribution (Takahashi et al., 2013; Takahashi, Nishikawa, & Takakura, 2017). They tracked the exosomes for 240 min, and showed that systemically administered exosomes have very short half-lives ($t_{1/2}$ ~ 2 min, and less than 5% of the administered exosomes remained in the serum 5 min post-iv injection) and the exosomes kinetics was first to the liver and then to the lungs (Figure 2).

Lai et al. engineered a membrane-bound variant of Gluc fused to a biotin acceptor peptide, for exosome labeling (Exosomes derived from human embryonic kidney [HEK] 293T cells). They conducted whole body bioluminescence imaging via combined 2D and 3D optical tomography (IVIS) and visualized the exosomes in the spleen 30 min post-iv injection of exosomes. They also reported that the highest signal was detected 1 min post-Coelenterazine substrate injection, followed by a rapid loss of signal within 5 min thereafter (C. P. Lai, Tannous, & Breakefield, 2014b). Van der Vos et al. used a different labeling agent and approach to visualize the RNA transfer of glioblastoma (GBM)-derived exosomes within the brain. They used GBM cells that were transduced with lentivectors encoding Fluc, mCherry, and palmtdTomato, and conducted in vivo imaging of the process every 5 min over 30 min, using multiphoton intravital microscopy (MP-IVM). Using this method they were able to elucidate one of the mechanisms by which GBM cells influence their microenvironment—through the active transferring of miRNAs by exosomes (van der Vos et al., 2016). Rluc reporter gene was used by Gangadaran et al. to determine whether exosomes preferentially target their parent cells. Anaplastic thyroid cancer (CAL62) cell-derived exosomes were labeled and administered iv to cancer model mice, a combined 2D and 3D optical tomography (IVIS Lumina III) imaging system was used for imaging 5, 30, 60, and 120 min postinjection. They revealed that exosomes accumulated in the lungs of mice without tumor xenograft, and in the tumor of CAL62 tumor-bearing mice and, within 30 min after injection, significant BLI signals were detected in the tumor (Gangadaran et al., 2018).

Taken together, the BLI technique provides a full-body picture of exosome biodistribution, and since the labeling is intrinsic, the exosome tracking is highly reliable. BLI also provides data on the process the exosomes are involved in, such as revealing the mechanisms by which cells influence their microenvironment through exosomes (van der Vos et al., 2016). However, there is a need to inject a substrate before each imaging session and the half-life of the substrate (such as Coelenterazine) is extremely short (up to 5 min, with a signal peak at 1 min postinjection), which necessitates a very fast and accurate imaging system (C. P. Lai, Tannous, & Breakefield, 2014b). Moreover, any in vivo imaging which is based on

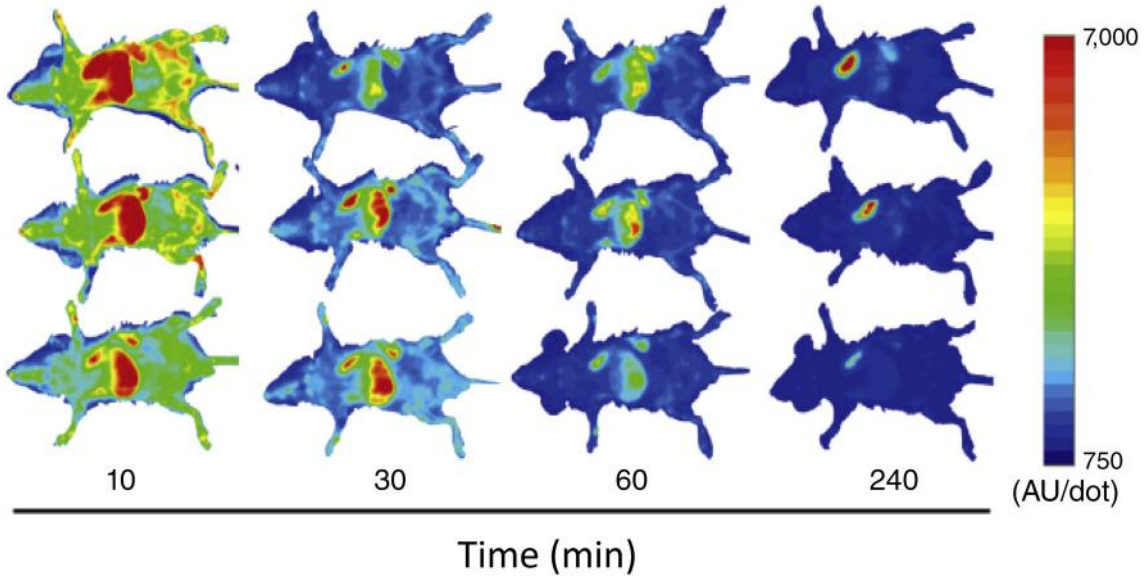

FIGURE 2 Kinetics of GLuc after intravenous injection of GL-labeled B16-BL6 exosomes into mice. Balb/c mice received intravenous injections of exosomes collected from pCMV-gLuc-lactadherin-transfected B16BL6 cells. B16-BL6 exosomes expressing gLuc were imaged 10, 30, 60, and 240 min postexosome injection through a bolus intravenous injection of Coelenterazine (Reprinted with permission from Takahashi et al. (2013). Copyright 2013 Elsevier)

fluorescence is limited in resolution and penetration depth so it cannot give a clear picture of exosome location in deep body structures (most studies rely on ex vivo imaging to validate the in vivo results). Therefore, this technique is mostly suitable for preclinical research, and will not be easily translated to the clinic (O'Neill, Lyons, Gallagher, Curran, & Byrne, 2009). Compared to FLI, BLI has an extremely low signal-to-noise ratio, due to a negligible auto luminescence in mammalian tissue. In particular, bioluminescence reporters are useful tools to study temporal properties of minute biological processes owing to their sensitivity, low background and independence from an excitation source to emit light (Frangioni & Hajjar, 2004; Villa et al., 2010).

1.4.2 | Tracking of exosomes using nuclear imaging

Nuclear imaging, involving the application of radioactive substances in the diagnosis and treatment of diseases, is broadly used for cellular imaging. Radionuclides emit radiation which can be detected in vivo using a special camera. Though radionuclides have very short half-lives, which necessitate relatively short periods of imaging and tracking, nuclear imaging enables visualization of deep structures and organs with its improved sensitivity and tissue penetration ability (Liu et al., 2012; Wester, 2007). In this method, the labeling agents are introduced directly to the exosomes through incubation, and 3D images are usually obtained using single-photon emission CT (SPECT) or positron emission tomography (PET) that can be combined with anatomical imaging, such as CT or MRI to improve its ability to localize the exosomes.

Smyth et al. (2015), who used fluorescence for imaging dox-loaded exosomes vs. liposomes accumulation within tumors and were discussed previously, also added radiolabeling of exosomes with Indium-oxine (^{111}In-oxine) to conduct ex vivo analysis of the bio-distribution 24 hr post-iv injection. This radiolabeling revealed the rapid blood clearance of both PC3 exosomes and MCF-7 exosomes in PC3 tumor bearing nude mice. Less than 5% of the injected dose remained 3 hr post-injection for both exosome types. Interestingly, the rapid clearance of the exosomes was similar for tumor bearing mice and healthy mice. Hwang et al. radiolabeled macrophage-derived exosomes with ^{99m}Tc-HMPAO and successfully imaged at 0.5, 3, and 5 hr post-iv injection. Imaging results demonstrated high radioactivity in the liver and spleen and no uptake in the brain, which is different from the bio-distribution of the radiolabeling agent alone (Figure 3; Hwang et al., 2015). ^{99m}TC radioisotope was also used by Varga et al. to develop a method for labeling of erythrocyte-derived exosomes using the ^{99m}Tc-tricarbonyl complex. They revealed that iv administered radiolabeled exosomes mostly accumulated in the liver and spleen between 1 and 2 hr postadministration.

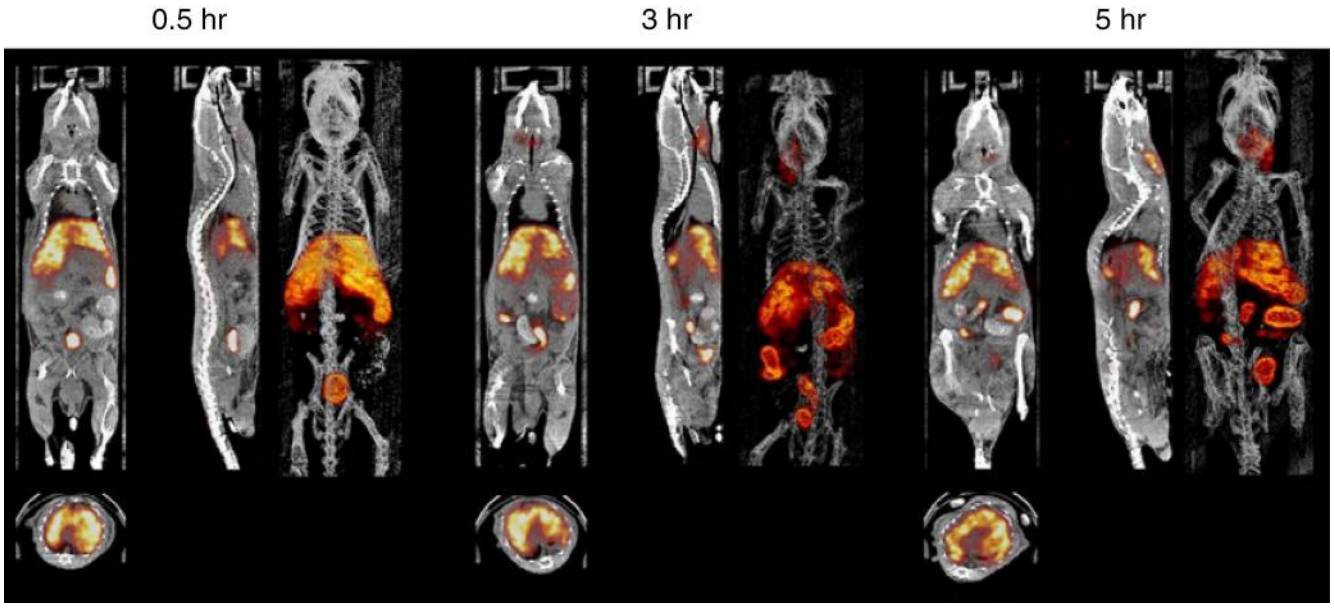

FIGURE 3 In vivo single-photon emission computed tomography/computed tomography (SPECT/CT) images of ^{99m}Tc-HMPAO labeled exosomes postintravenous injection. Images were acquired at 30 min, 3, and 5 hr in BALB/c mice. The SPECT/CT imaging shows a significant uptake of radiolabeled exosomes in the liver, salivary glands and intestine up to 5 hr postadministration (Reprinted with permission from Hwang et al. (2015). Copyright 2015 Creative Commons Attribution 4.0 International License)

Gangadaran et al. used a different imaging modality for tracking labeled exosome-mimetics derived from red blood cells labeled with ^{99m}Tc. They conducted in vivo imaging of the labeled exosomes in mice using gamma camera at 1 and 3 hr post-iv injection. Results showed higher uptake in the liver and spleen, and no uptake in the thyroid, while free ^{99m}Tc signal was detected mostly at in the thyroid, stomach, and bladder. They confirmed their radiolabeling results using both fluorescence and immuno-FLI (Gangadaran et al., 2018). A recent paper by Faruqu et al. used a different isotope (^{111}Indium) and a different labeling approach: intraluminal labeling (entrapment of ^{111}Indium via tropolone shuttling) and membrane labeling (chelation of ^{111}Indium via covalently attached bifunctional chelator). They radiolabeled melanoma cell-derived exosomes (B16F10) for bio-distribution analysis in immunocompetent and immunodeficient mice. Whole body imaging of iv injected membrane-labeled B16F10 exosomes, revealed that the exosomes had a substantial lung accumulation at 1 hr post-tadministration, which then continued to decrease over time from 4 to 24 hr (Faruqu et al., 2019).

Taken together, radiolabeling gives a clear, deep-penetrating, and whole-body imaging of the exosomes, and can be combined with CT to provide simultaneous anatomical imaging. However, many radionucleides have very short half-lives—most studies tracked exosomes using ^{99}Tc between 1 and 5 hr, while only ^{111}In allowed a longer tracking period of up to 24 hr.

1.4.3 | Tomography

Tomography techniques are considered to be efficient and noninvasive methods, are widely used, and can be easily translated to the clinic. Tomography techniques are suitable for longitudinal tracking and localizing of exosomes and for visualizing exosomes over time; tomography methods require the exosomes to be labeled with nanoparticles as labeling agents.

Tracking of exosomes using magnetic imaging

MRI has advantages such as being radiation-free and having high spatial resolution (Crabbe et al., 2010). In order to be detectable by MRI, exosomes must be labeled with magnetic contrast agents, such as superparamagnetic iron oxide nanoparticles (SPIONs; Busato et al., 2017; Motiei et al., 2019; Vegerhof et al., 2015). Hu et al. demonstrated for the first time that exosomes loaded with 5 nm SPIONs cargo can be imaged and tracked using the MRI modality in vivo. SPIONs were successfully encapsulated within melanoma cell-derived exosomes (B16B10) using electroporation. MRI imaging revealed that the exosomes migrated from the foot pad to the lymph node up to 48 hr post-iv injection (Figure 4). They concluded that exosomes preferentially home to certain resident structural regions of the lymph node (Hu et al., 2015).

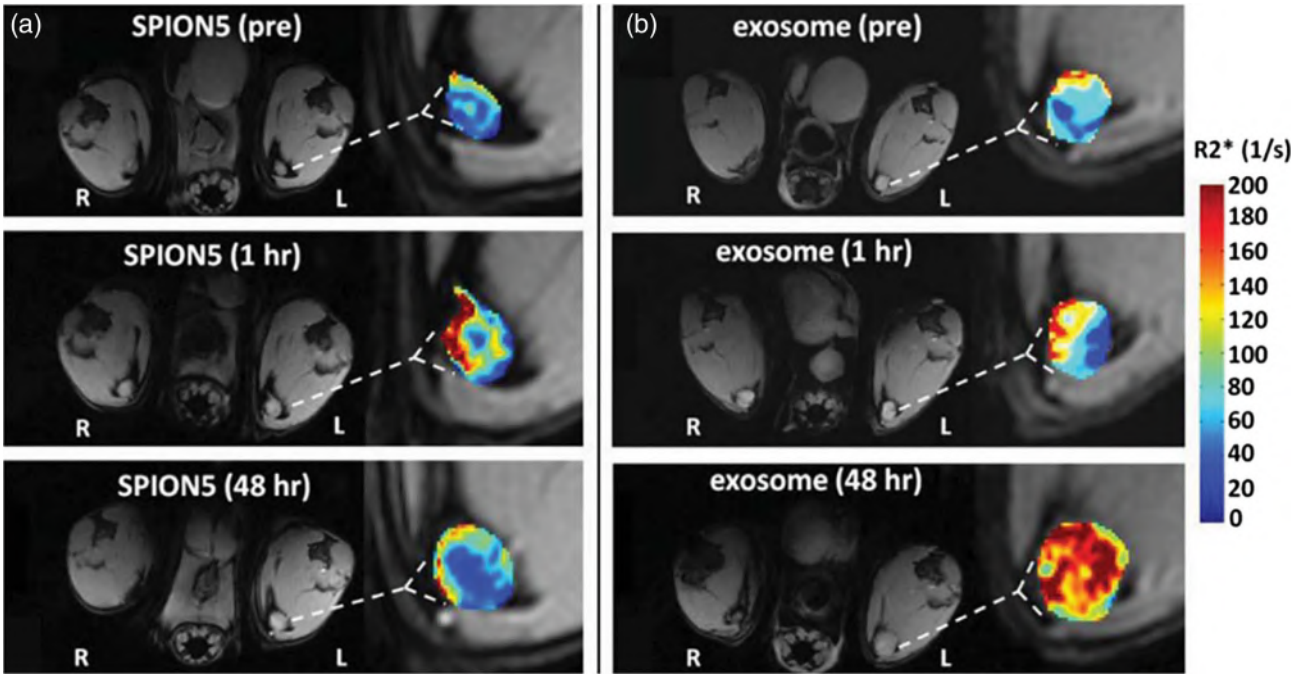

FIGURE 4 In vivo MRI of SPION5 loaded melanoma exosomes. (a) Free SPION5 and (b) SPION5 loaded exosome migrated to ipsilateral (left, L) and contralateral (right, R) popliteal lymph nodes (PLN) as visualized by T1-weighted images with R2* mapping of PLN ipsilateral to the injection site (Reprinted with permission from Hu et al. (2015). Copyright 2015 Creative Commons Attribution 4.0 International License)

Ultrasmall superparamagnetic iron oxide nanoparticles (USPION) were used by Busato et al. to label adipose stem cells before vesicles extraction. The isolated exosomes retained the nanoparticles and could be visualized by MRI 1 h post-intramuscular injection (Busato et al., 2016). Jc Bose et al. have exploited tumor cell-derived extracellular vesicles for cancer-targeted delivery of miRNA therapeutics to the tumor combined with phototherapy treatment. They labeled the exosomes by fusing gold–iron 70 nm nanoparticles (GIONs) with the exosomes via a top-down process involving extrusion through a 100 nm porous membrane. T2-weighted MR imaging was conducted on day 12 postsystemic administration. The T2-weighted gradient echo imaging showed significant accumulation of exosomes in the tumor, as evidenced by the increased susceptibility effect, as well as T2 hypointensity in the areas of the tumor mass (Jc Bose et al., 2018). Magnetic particle imaging (MPI) is another developing noninvasive, diagnostic, in vivo tomographic imaging technique used for tracking SPIO nanoparticles (E. Y. Yu et al., 2017; Zheng et al., 2015). MPI directly identifies the intense electronic magnetization of SPIOs, and not indirectly like MRI which detects the SPIOs' signal dropouts. It has significant advantages such as high sensitivity and specificity without ionizing radiation along with the no signal decrease with tissue depth (Jung et al., 2018). MPI was used to monitor exosomes in vivo by Jung et al. (2018) that developed an exosome platform which targets regions of tumor hypoxia. Four types of exosomes (generated under hypoxic/normoxic conditions, and with/without exposure to X-ray radiation), isolated from MDA-MB-231 human breast cancer cells, were modified to carry SPIO nanoparticles through indirect labeling of their parent cells. The exosomes were also modified to carry Olaparib—a PARP inhibitor, and were labeled with fluorescence DiO as well. Their results demonstrated that Tumor hypoxic cells preferentially take up exosomes released by hypoxic cells, and "hypoxic" exosomes were successfully modified for MPI imaging and in vivo drug delivery. MPI imaging of iv injected exosomes imaging showed that exosomes accumulated primarily in the liver and MPI imaging of IT injected exosomes verified the successful administration within the tumor.

Taken together, MRI enables longitudinal imaging—findings show imaging up to 12 days after iv injection (Jc Bose et al., 2018), with high resolution and maximal penetration into deep structures. It must be taken into account that the nanoparticles may not all be contained within the exosomes for the entire duration, and the images may reflect entry of SPIONs into the cells after exosome fusion, thus this modality does not necessarily provide information on exosome final fate.

Tracking of exosomes using CT

CT is among the leading imaging techniques applied in the field of biomedical imaging (Shilo, Reuveni, Motiei, & Popovtzer, 2012). CT is characterized by high temporal and spatial resolution, and is among the most convenient imaging tools used in hospitals to date in terms of availability, efficiency, and cost. CT provides superior visualization of bone structures due to the inherent contrast between electron-dense bones and the more permeable surrounding soft tissues (Meir, Shamalov, et al., 2017; Shwartz et al., 2017). Therefore, CT has been used for cell tracking while combined with nanoparticles as labeling agents (Betzer et al., 2014; Betzer et al., 2015; Betzer, Meir, Motiei, Yadid, & Popovtzer, 2017; Betzer, Shilo, Motiei, & Popovtzer, 2019; Betzer, Shilo, et al., 2017; Hazkani et al., 2017; Meir, Betzer, et al., 2017; Meir, Betzer, Barnoy, Motiei, & Popovtzer, 2018; Popovtzer, 2014; Shilo et al., 2015; Shwartz et al., 2017).

Recent papers used CT for tracking intranasally (IN) administered gold-labeled exosomes within the brain (Betzer, Perets, et al., 2017; Betzer, Perets, Barnoy, Offen, & Popovtzer, 2018; Perets et al., 2019). A protocol for direct labeling of exosomes with gold nanoparticles (GNP) was developed. MSC-derived exosomes were labeled directly through incubation with glucose-coated 5 nm GNP and tracked for the duration of 96 hr in mice models for Stroke, Alzheimer's, Parkinson and autism (Figure 5). To analyze the distinct distribution patterns of GNP-labeled exosomes within pathological brains, CT signal intensity was noninvasively quantified in various regions of interest. This imaging technique provided insight about the migration mechanism of the exosomes towards the pathology regions, with the involvement of the innate immune system.

Taken together, CT provides a widely available imaging modality able to track exosomes with good resolution throughout an entire body with excellent penetration. However, this modality requires direct exosome labeling, with all of the drawbacks associated with the process, such as not knowing whether the signals that appear in the images refer to actual exosomes or leaked or transferred contents. There also exists the ionizing radiation issue associated with any CT imaging.

1.4.4 | Tracking of exosomes using photoacoustic imaging

Photoacoustic imaging (PAI) is a recently developed hybrid biomedical imaging modality, it is based on the photoacoustic effect and combines ultrasound imaging with optical imaging. The contrast agent in the biological sample absorbs the pulsed

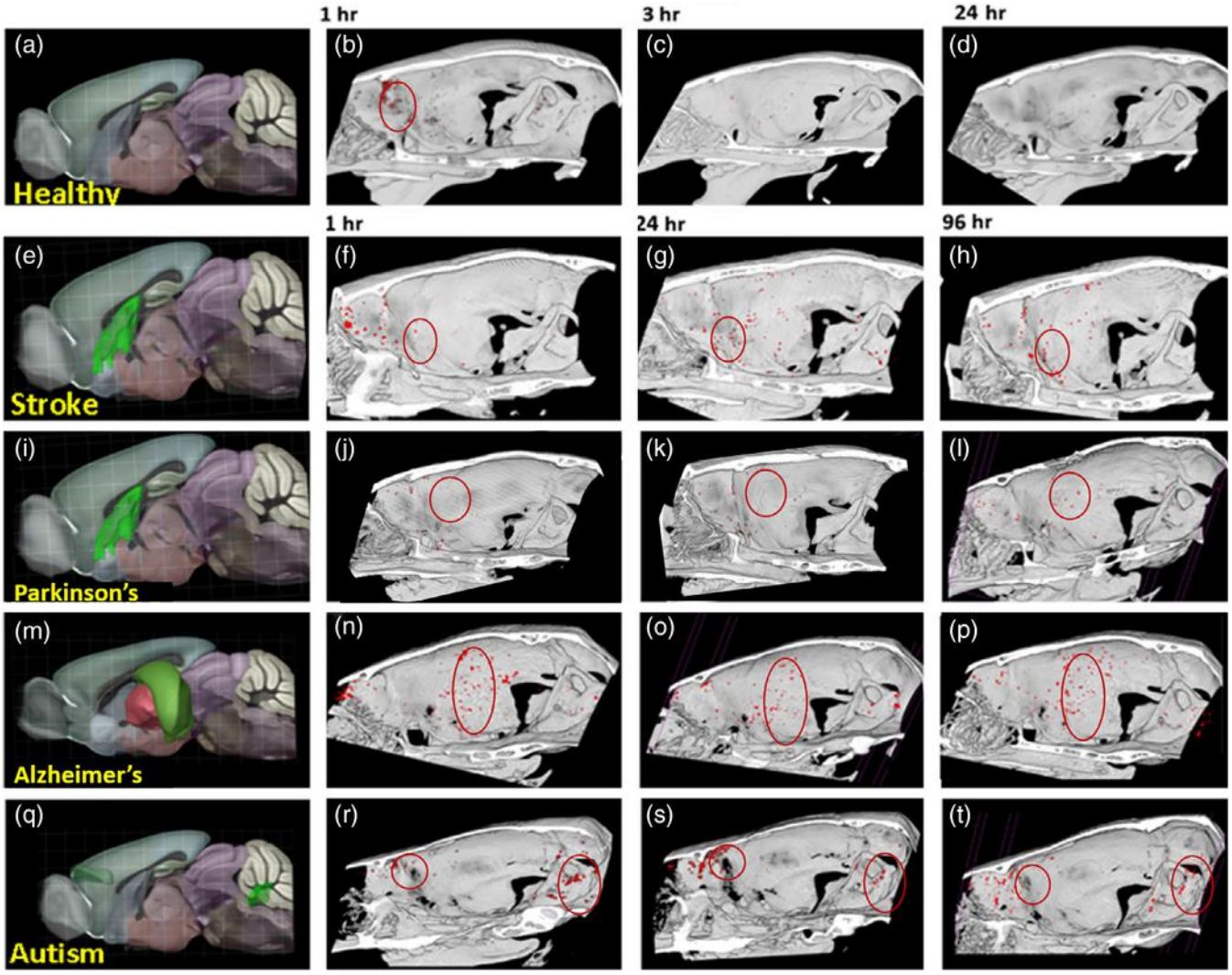

FIGURE 5 (a) Longitudinal in vivo computed tomography (CT) imaging of homing and accumulation of exosomes within the brain. (b–d) Healthy mice. CT signal, indicating presence of gold nanoparticle-labeled exosomes, is found mainly in the olfactory bulb at 1 hr, and cleared by 24 hr. (f–h) Stroke model: the exosomes CT signal was located in mouse striatum up to 96 hr. (j–l) Parkinson's disease model: exosomes signal was located in the striatum at 96 hr. (n–p) Alzheimer's model: exosomes signal was located in the hippocampus up to 96 hr. (r–t) Autism model: exosomes signal was located in the cerebellum and prefrontal cortex up to 96 hr. (a, e, i, m, q). Brain section images adopted from Allen Mouse Brain 3D atlas, showing the lesioned/pathological area in green (Reprinted with permission from Perets et al. (2019). Copyright 2019 American Chemical Society)

laser radiation energy and converts it into an acoustic signal, which is then measured and visualized by a scanning transducer (Chen et al., 2017). Thus, PAI is an attractive noninvasive imaging modality that combines the advantages of high spatial resolution and deep penetration of ultrasound imaging with the high-contrast of optical imaging (Li, Chen, Du, & Chen, 2018; Xu & Wang, 2006; Gong et al., 2019).

Piao et al. (2018)) used noninvasive BLI and PAI to monitor tumor growth and axillary lymph-nodes (LN) metastasis in orthotopic triple-negative breast cancer (TNBC) models. They used RFP-tagged exosomes (labeled indirectly through parent cells) and found out that iv injection of TNBC cell-derived exosomes promoted primary tumor growth and axillary LN metastasis (Figure 6). These results suggest breast cancer cell-derived exosomes stimulate macrophage polarization that creates favorable conditions for LN metastatic processes in TNBC. Engineered exosomes (from red blood cells membrane) that are H_2O_2-responsive due to labeling with graphene quantum dot nanozyme were developed by Ding et al. (2019) as biological contrast agent carriers for PAI in order to improve tumor visualization and diagnosis. These exosome-like vesicles accumulated in the tumor area 2 hr post-iv injection and the PA signal increased up to 8 hr.

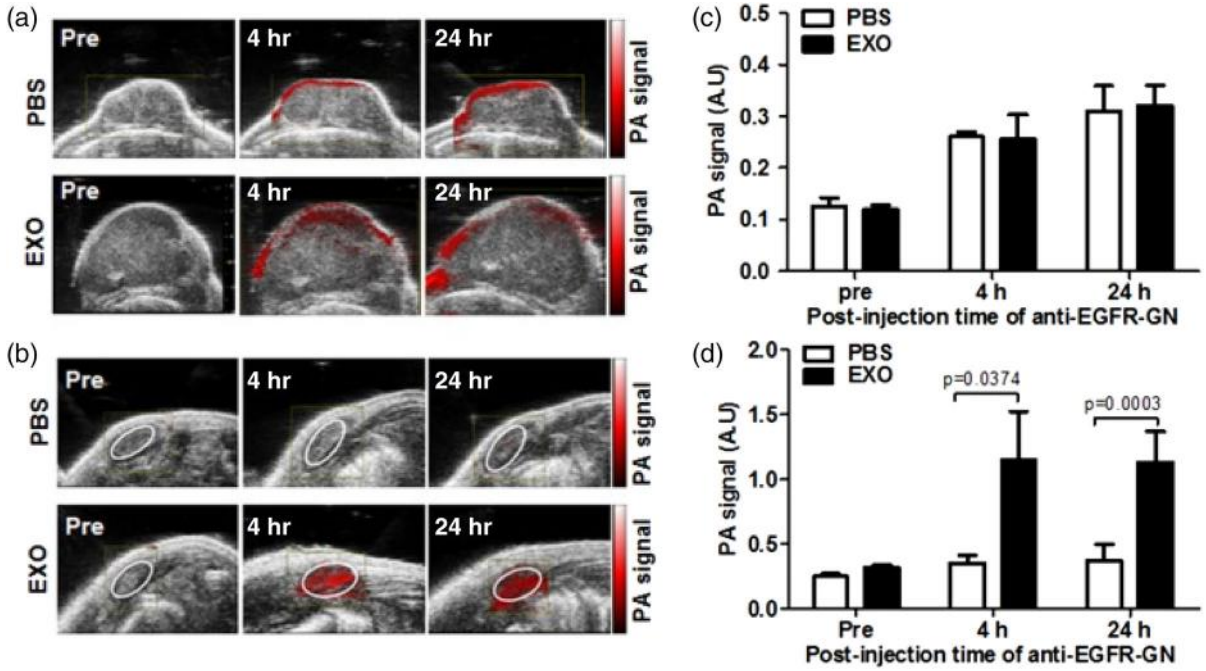

FIGURE 6 Photoacoustic imaging (PAI) of primary tumor growth and axillary lymph-nodes metastasis following intravenous (iv) injection of cancer cell-derived exosomes in triple-negative breast cancer models. (a) and (b) Representative ultrasound-guided PAI of mice that were injected iv with phosphated-buffered saline or exosomes before, 4 and 24 hr after intratumor injection of anti–EGFR-GN. (c and d) PA signals (mean ± *SE*) measured from primary tumors (c) and axillary lymph-nodes (d). Exosomes reached the tumor area and promoted its growth (Reprinted with permission from Piao et al. (2018). Copyright 2018 Creative Commons Attribution License 3.0)

2 | CONCLUSIONS

In this review, we examined studies that have, to date, tracked exosomes in vivo. Research using these methodologies is pioneering, but the importance for exosome research is that these vesicles are indeed trackable within a living body. These various tracking methods can elucidate much of the unknown behavior and function of exosomes, differentiate between different exosome types, define the role exosomes play in disease, explore their capabilities in navigating throughout the body in terms of active distances and barrier penetration, and reveal how they interact with cells. We have discussed different methods of labeling and imaging exosomes, and how these different methods lead to differing benefits or drawbacks. Moreover, research indicated that exosome biodistribution and in vivo migration can also be affected by the delivery route that was used for administration. Betzer, Perets, et al. (2017) used both iv and IN administration delivery routes and demonstrated using CT that, for targeting the brain, IN administration was more effective than iv injection since IN administered exosomes show more widespread biodistribution and enhanced brain accumulation over time. Jung et al. (2018) used both iv and IT administration of exosomes to treat subcutaneous tumor, and MPI imaging revealed that iv injected exosomes were less suitable for tumor targeting since the exosomes accumulated primarily in the liver while IT injection entered the tumor directly and was shown to be significantly more effective. Any specific desired outcome would need due consideration in choosing the appropriate means of labeling, administering, and tracking. Moreover, a powerful tool for visualizing and shedding light on exosome behavior is using a multimodal bioimaging approach combining several modalities, each having its unique features and capabilities. For example, Shaikh et al. (2018) used CT, FLI, and MRI imaging for improving precise and early diagnosis of tumors. A multimodal approach provides the potential to make use of the advantages of each modality while reducing the overall drawbacks. Exosomes measureable by both CT and FLI can be tracked in vivo using CT, and then more precisely ex vivo with FLI (Guo et al., 2019). Also, this approach allows for self-validation, since a measured result can be confirmed by the other modalities (C. P. Lai, Mardini, Ericsson, et al., 2014; Yuki Takahashi & Takakura, 2015). Furthermore, it is important to keep in mind that some tracking modalities are mostly useful for preclinical studies (such as FLI and BLI), while others can easily be adapted from research to the clinic (such as CT, MRI, or PET).

Despite the positive outlook, it is essential to note several key aspects that should be further explored in future studies. First, the lack of standardization in exosome isolation, purification, and characterization procedures hampers direct

comparison between different findings. Additionally, different isolation and purification methods which affect exosome purity and function may consequently have an impact on their in vivo bio-distribution. These issues are manifested by the lack of stating the amounts of exosomes injected, or the marking efficiency of exosomes, in many studies. There is therefore a challenge for future research to make the tracking of exosomes more quantifiable and thus allow for more measureable comparisons between the different tracking modalities. Up to this point there has also not been a study to link between the traverse of exosomes across the body to their therapeutic abilities. A future study could incorporate artificial intelligence and machine learning in order to analyze such information and so be able to predict, soon after exosome treatment is started and with the proper choice of imaging modality, whether the movements of the exosomes reveals a high or low chance of success for the treatment. Further developments could also lead to greater understanding of the biological functionality of exosomes, thus allowing the use of the exosomes for delivery of medications in a targeted manner. They would be able to combat a myriad of diseases and treat specific patients and their individual needs in mind.

Exosomes offer many therapeutic promises, and many tracking methodologies have been developed to explore their functionality. Future research that is sure to come will enable to construct a more complete picture of their abilities and limitations.

ACKNOWLEDGMENTS

This work was partially supported by the Israel Science Foundation (ISF) (749/14), the Israel Science Foundation Joint NSFC-ISF Research Grant (2533/17) and the Ministry of Science and Technology Israel-China cooperation. We would like to thank the council for higher education for supporting O.B. with postdoctoral fellowship for outstanding women in science, and the Ministry of Science, Technology and Space Israel for granting her the industry-academy postdoctoral scholarship (3-15677).

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Oshra Betzer: Conceptualization, lead; formal analysis, lead, investigation, lead; writing-original draft, lead. Eran Barnoy: Writing-review and editing, equal. Tamar Sadan: Conceptualization, equal; writing-review and editing, equal. Idan Elbaz: Investigatio, supporting. Cara Braverman: Investigation, supporting. Zhuang Liu: Conceptualization, supporting; supervision, supporting. Rachela Popovtzer: Conceptualization, supporting; resources, lead; supervision, lead.

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How to cite this article: Betzer O, Barnoy E, Sadan T, et al. Advances in imaging strategies for in vivo tracking of exosomes. *WIREs Nanomed Nanobiotechnol*. 2019;e1594. https://doi.org/10.1002/wnan.1594