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Quantifying nanoparticle delivery: challenges, tools, and advances

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This review explores challenges and methods for quantifying nanoparticle delivery in therapeutic applications. We discuss three main approaches: (1) functional readouts that assess therapeutic effects post nanoparticle administration, (2) nanocarrier tracking that directly monitors the nanoparticle localization, and (3) cargo tracking that infers nanoparticle localization by measuring encapsulated agents or attached surface tags. Reanalysis of the Wilhelm et al. Cancer Nanomedicine Repository dataset found mixed quantification methodologies, which could cause misleading conclusions. We discuss potential pitfalls in each quantification approach and highlight recent advancements in novel technologies. It is important that researchers select appropriate quantification methods based on their objectives and consider integrating multiple approaches for a comprehensive understanding of *in vivo* nanoparticle behavior to facilitate their clinical translation.

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Introduction

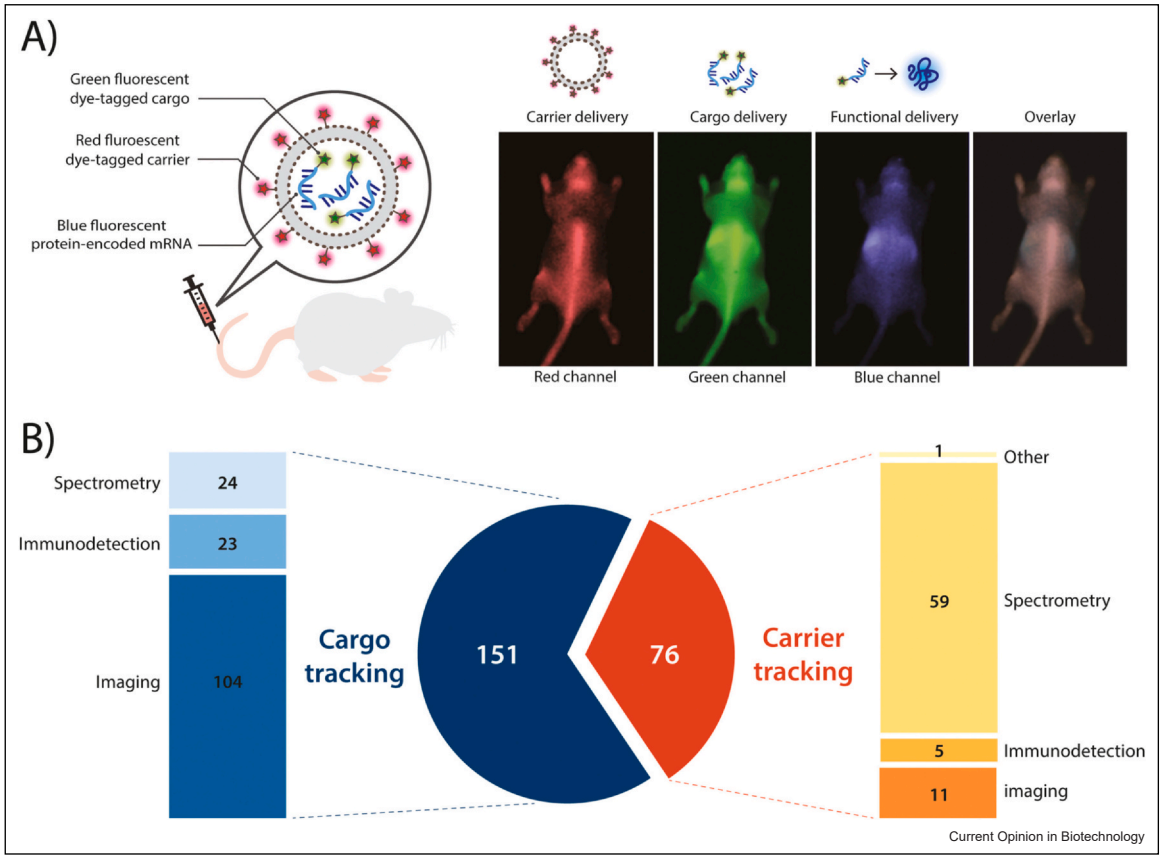
Nanoparticles show great promise for drug and gene delivery applications. Nanoparticle-based carrier systems can enhance the solubility of hydrophobic drugs, extend blood circulation time, control temporal release of drugs, and deliver cargo to specific cell types. Nanoparticle delivery quantification is important because nanoparticles need to access their intended target site at sufficient dosage to elicit their therapeutic function [1]. There are three main modes of quantifying nanoparticle delivery: (1) functional readouts, (2) nanocarrier tracking, and (3) cargo tracking (as shown in Figure 1a). It is inappropriate to compare different nanoparticle delivery system designs when different measurement modalities are used. For example, in cancer nanomedicine, many meta-analyses have been conducted to evaluate tumor delivery of different nanoparticle designs. Notably, Wilhelm et al. systematically analyzed hundreds of nanoparticle tumor delivery datasets and found that only 0.7% (median) of systemically administered nanoparticles reached the tumor [2]. Other groups have refined or updated the inclusion criteria [3,4], considered additional measured parameters [5], or performed more comprehensive pharmacokinetic modeling [3,4,6] to quantify delivery efficiency more accurately. However, a significant conceptual gap still exists since the quantification methodologies are vastly different throughout literature. It is known that nanocarrier distribution can be different from its cargo distribution, and delivery of the therapeutic cargo may not yield sufficient observable functional effects [7–9], as shown schematically in Figure 1a. We reanalyzed Wilhelm et al. dataset and recategorized the entries as carrier or cargo tracking, and further subcategorized by analytical quantification techniques. Functional readout measurements were excluded from the initial dataset already. Our reanalysis (Figure 1b) shows that ~66% of entries used cargo tracking versus ~34% used carrier tracking methods. Imaging-based techniques were preferred in cargo tracking entries (~69%), whereas spectrometric techniques were majorly used in carrier tracking (~78%). As such, it is evident that the delivery efficiency values and pharmacokinetic parameters derived from these nanoparticle tumor delivery meta-analyses may be misleading due to the mixed quantification methodologies used, akin to comparing apples to oranges.

Functional readouts

Functional readouts encompass methodologies for measuring therapeutic activity, outcomes, and efficacy post nanoparticle administration as summarized in Figure 2a. In cancer nanomedicine, examples of functional readouts include survival, disease remission, and tumor growth inhibition. Functional readout examples in gene delivery include transgenic protein expression or gene expression profile changes.

Transgenic reporter animals are becoming increasingly popular and useful in functional readout approaches. These animals are genetically engineered to express

Figure 1



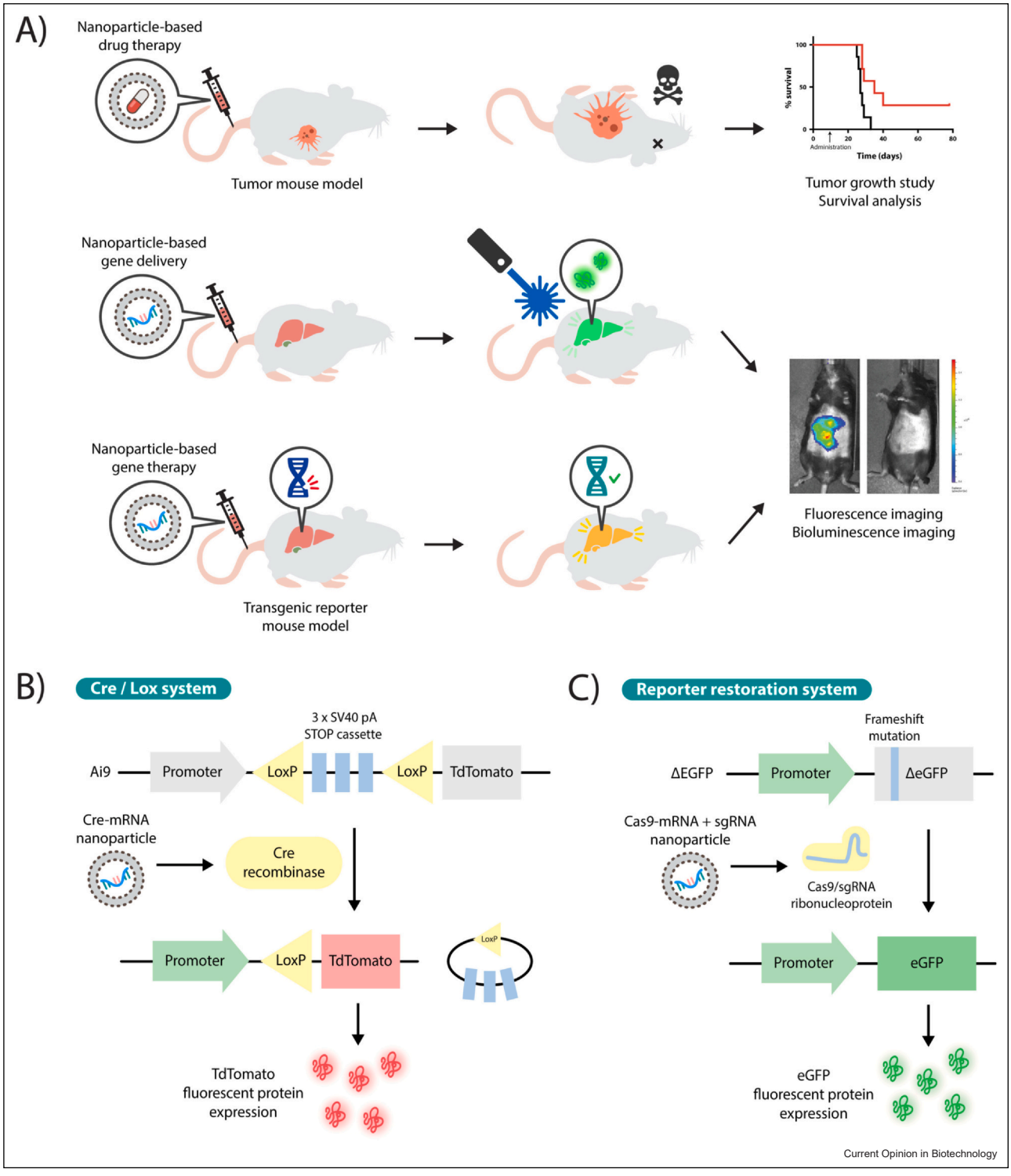
Reanalysis of Cancer Nanomedicine Repository data based on quantification methodology. **(a)** Example of using three modes of quantifying nanoparticle delivery — a mouse is injected with a nanoparticle where the nanocarrier is tagged with a red fluorescent dye, and the gene cargo that encodes for a blue fluorescent protein is tagged with a green fluorescent dye. *In vivo* animal imaging using the red fluorescence channel would be cargo-tracking, using the green fluorescence channel would be cargo-tracking, and using the blue fluorescence channel would be functional readout. Notably, overlaying all three fluorescent channels shows that the carrier, cargo, and expressed functional protein do not have the same biodistribution and delivery in the animal. **(b)** Reanalysis of Cancer Nanomedicine Repository information from Wilhelm et al. by nanoparticle delivery quantification method and analytical technique [2].

fluorescent, bioluminescent, or other detectable markers upon a specific trigger. They are amenable to *in vivo* longitudinal tracking methods and *ex vivo* quantification or visualization techniques [10]. Two common types of transgenic reporter mice used to quantitate nanoparticle delivery are (1) Cre/lox systems and (2) reporter protein restoration systems as summarized in Figure 2b and c.

In Cre/lox systems, Cre recombinase is delivered either as a gene or protein using nanoparticles. Cre recombinase excises out the stop cassettes between loxP sequences that prevent downstream transcription from a strong promoter. This subsequently leads to production of a reporter protein in the specific cells to mark where nanoparticle delivery occurred. Ai9/Ai14 mice and mTmG mice are common Cre/lox reporter mice used for quantifying nanoparticle delivery. Ai9/Ai14 mice are also often used to evaluate nanoparticle-based delivery of CRISPR–Cas genome editors, where sgRNA guides direct Cas9 to the 5′ and 3′ loxP sites to excise the stop cassette and turned-on expression of the tdTomato reporter [11,12]. In reporter restoration systems, a mutation in the coding sequence disrupts reporter protein function. Upon nanoparticle administration of genome editing tools, gene editing and repair correct the mutation to restore reporting activity. Examples of reporter restoration animals include the ΔeGFP [13] mouse with a frameshift mutation in eGFP transgene, and the LumA mouse with a nonsense mutation in the luciferase transgene [13].

Several caveats must be considered when assessing delivery through reporter expression. First, fluorescence reporter mRNA may not yield sufficient expression detectable above background fluorescence *in vivo* [14]. Additionally, reporter expression timing must be factored in as it typically takes hours after nanoparticle entry for expression and function initiation in cells [15],

Figure 2



Functional readouts for quantifying nanoparticle delivery. **(a)** Examples of functional readouts for nanoparticle-based drug and gene delivery. **(b)** Schematic of the Cre/lox reporter system. **(c)** Schematic of the reporter protein restoration system.

which results in poor temporal resolution for quantifying nanoparticle delivery. Furthermore, reporter expression can persist over an extended period *in vivo*, but its level is influenced by many external factors unrelated to nanoparticle delivery. For example, using the single-sgRNA system with Cas9 editing in Ai9/Ai14 transgenic mice can randomly remove 1 or 2 of the 3 stop cassettes, leading to varied TdTomato expression and fluorescence output [16,17]. Moreover, there can be inaccuracies in genetic recombination in Cre/lox systems after Cas9 editing and Cre-mediated recombination, which can decouple reporter expression from nanoparticle delivery [18]. Reporter mice can also misrepresent delivery due to variations in cell types, their division rates, and preferred DNA repair mechanisms to affect reporter protein expression [19]. In summary, transgenic mice models offer valuable insights into nanoparticle function, but provide only semiquantitative nanoparticle delivery data. They can assist in studying nanoparticle behavior but should not be solely relied upon to make definitive claims about targeted delivery or reduced off-target effects.

Carrier tracking

Carrier tracking involves analyzing the distribution and localization of nanocarriers based on the intrinsic physical properties of the tracked nanomaterial. The main carrier tracking methods in vivo include magnetic resonance imaging (MRI), ultrasound, near-infrared (NIR) fluorescence imaging, and computed tomography (CT) as shown in Figure 3a.

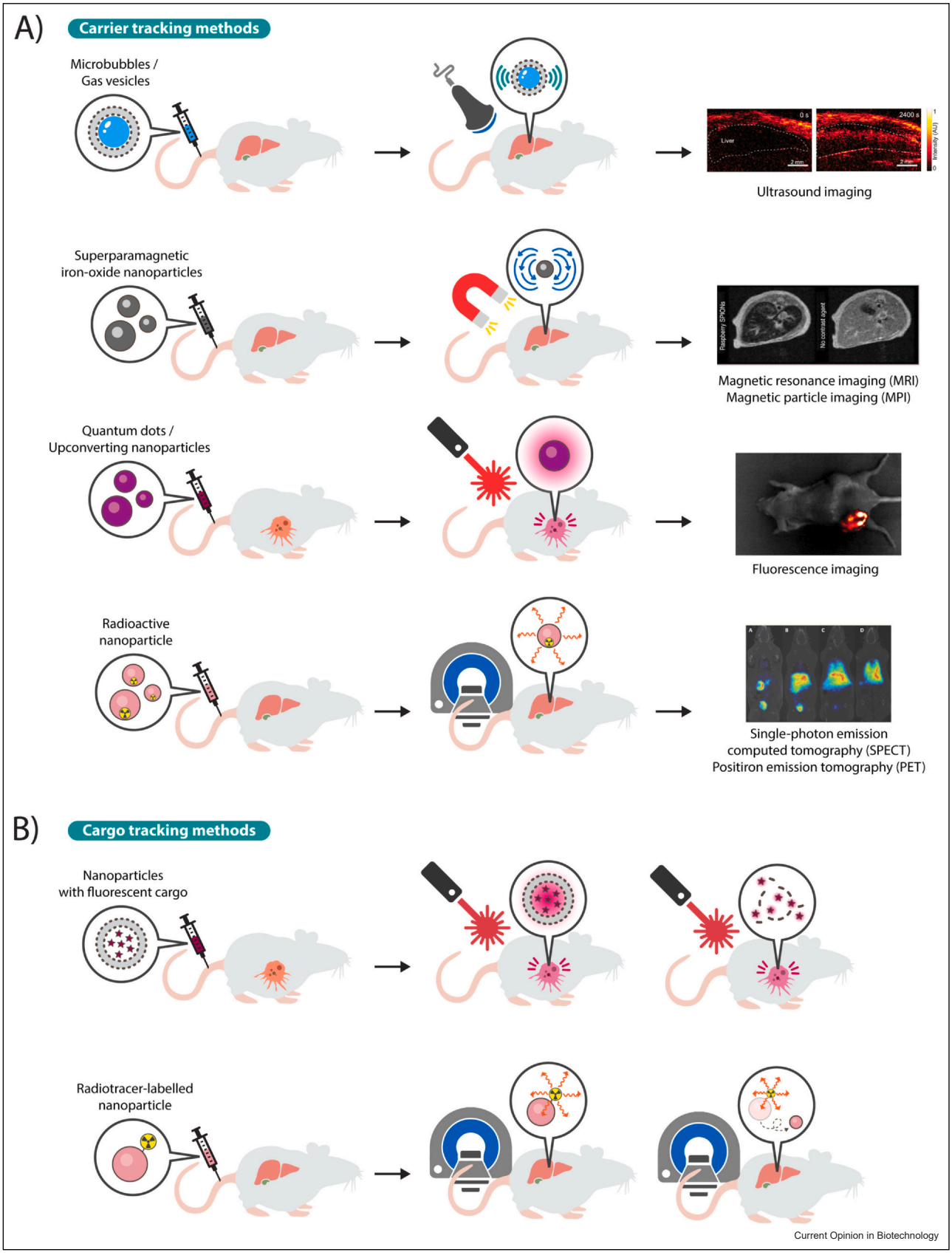
MRI is commonly used to track superparamagnetic iron oxide nanoparticles (SPIONs) because the magnetic moments of the nanoparticles' constituent iron oxide crystals can align with the MRI's magnetic field. Clinically approved SPION formulations, including Feridex® and Feraheme™, can be tracked using MRI *in vivo* as negative contrast agents [20]. MRI tracking has sufficient deep tissue penetration for clinical use, but it is not linearly quantitative for nanoparticle concentration due to magnetic field inhomogeneities and intrinsic background magnetic signals [21]. Emerging magnetic particle imaging (MPI) techniques offer improved *in vivo* SPION localization [22,23] by providing enhanced sensitivity and specificity since it generates positive contrast [23]. Additionally, MPI directly detects the electronic magnetization of SPIONs, surpassing the nuclear magnetization of protons seen in MRI by a factor of 10^8, thereby allowing the detection of subnanomolar iron concentrations from nanoparticles [23,24]. Ongoing efforts aim to upscale the size and availability of magnetic particle imagers to make them more accessible in clinical settings for patient use [22,24].

Although it has lower tissue penetration compared with MRI, ultrasound imaging is also a valuable tool for monitoring nanocarriers *in vivo* due to its clinical accessibility [25]. Nanoparticles in ultrasound imaging create contrast by scattering and reflecting acoustic waves due to their high-impedance mismatch with surrounding tissues [26]. Micro- and nanobubbles are commonly used ultrasound contrast agents that generate strong echogenic responses [27]. Recent innovations in ultrasound-based tracking include the development of new acoustically sensitive nanocarriers and evaluating their contrast using higher-order harmonics and phase shift [28]. For example, novel acoustic gas-filled nanoscale protein nanostructures termed gas vesicles (GVs) have been developed with strong sound wave reflection capabilities [25,29]. Protein engineering can create GVs with distinct fundamental and harmonic responses, enabling multiplexed ultrasound imaging through spectral analysis [30]. Additionally, GVs with adjustable collapse pressure enhance ultrasound imaging sensitivity by controlling their intrinsic scattering pattern [30,31].

Another popular *in vivo* nanocarrier tracking methodology is NIR imaging, which uses NIR light-absorbing or NIR light-emitting nanoparticles as contrast agents [32]. Notably, NIR light can penetrate deeper into tissue compared with visible light due to reduced tissue scattering, absorption, and autofluorescence background [33]. Quantum dots and upconversion nanoparticles are commonly used with NIR imaging [34]. Recently, new strategies in signal analysis have harnessed the optical properties of NIR nanoparticles for delayed and persistent luminescence imaging [35]. Fan et al. engineered lanthanide-doped NIR nanoparticles with varying luminescence lifetimes spanning three orders of magnitude, enabling time-domain-multiplexed *in vivo* imaging in mice [35]. Additionally, Pei et al. developed X-ray-activated, lanthanide-doped nanoparticles with long emission times for high-contrast *in vivo* imaging without external illumination, thereby greatly reducing background noise from autofluorescence [36].

Positron emission tomography (PET) and single-photon emission tomography (SPECT) use radiolabeled nanoparticles for nuclear imaging [37]. PET uses positron-emitting isotopes, whereas SPECT uses gamma-emitting isotopes. These nuclear imaging techniques are highly sensitive to concentration but usually require X-ray computed tomography or MRI for anatomical reference [38]. Incorporating radioactivity into inorganic and polymeric nanoparticles directly can be achieved through various methods such as specific trapping or ion exchange of radionuclides into nanoparticle via coordination bonding, hot-plus-cold precursor synthesis, and proton beam activation [37,39]. For instance, Frellsen et al. trapped ^{64}Cu into gold nanoparticles for PET imaging to study nanoparticle surface modification

Figure 3


A)
Carrier tracking methods
Microbubbles / Gas vesicles
Ultrasound imaging
Superparamagnetic iron-oxide nanoparticles
Magnetic resonance imaging (MRI)
Magnetic particle imaging (MPI)
Quantum dots / Upconverting nanoparticles
Fluorescence imaging
Radioactive nanoparticle
Single-photon emission computed tomography (SPECT)
Positiron emission tomography (PET)
B)
Cargo tracking methods
Nanoparticles with fluorescent cargo
Radiotracer-labelled nanoparticle
Current Opinion in Biotechnology

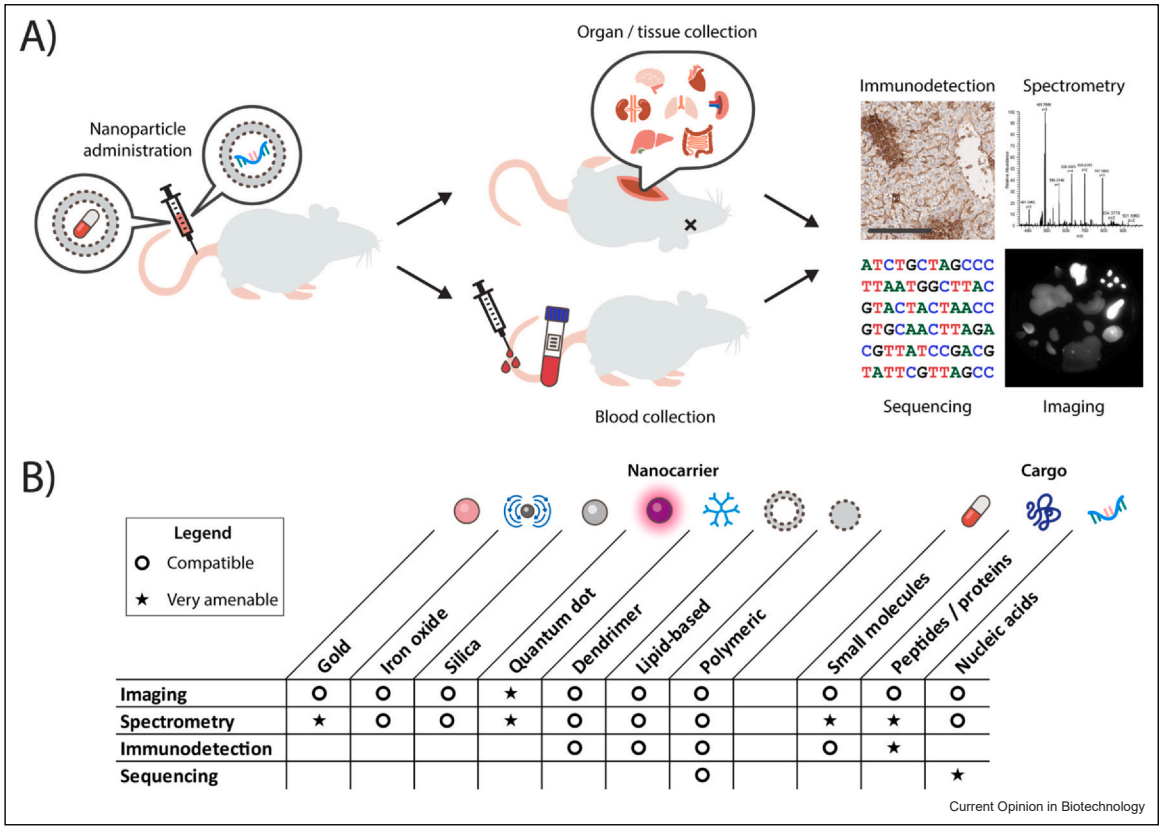
Carrier versus *cargo tracking methods for quantifying nanoparticle delivery*. **(a)** Examples of carrier tracking methods for nanoparticle-based drug delivery, including ultrasound imaging, magnetic resonance imaging, NIR fluorescence imaging, and nuclear imaging such as PET and SPECT. **(b)** Examples of cargo tracking methods for nanoparticle-based drug delivery and potential pitfalls.

effects on biodistribution and pharmacokinetics [40]. Zhao et al. doped gold nanoparticles with radioactive ^{199}Au for tracking tumor delivery via SPECT/CT imaging [41]. The practical use of intrinsic radioactive nanoparticles is limited due to safety concerns with prolonged radioactivity exposure to researchers and logistical time constraints with regard to radioactive decay throughout the entire synthesis, transport, storage, and experimental processes [37,38]. New imaging methods allowing simultaneous PET and SPECT *in vivo* imaging may further enhance nanoparticle quantification using nuclear imaging in clinical settings [42].

Cargo tracking

Cargo tracking involves inferring nanoparticle delivery by monitoring and measuring the presence of agents encapsulated inside nanoparticles or additional tags attached to their surface. Common encapsulated agents and conjugated tags include radiolabels, fluorescent dyes, DNA barcodes, drugs, and genes. However, this approach assumes that the cargo or tag remains coupled to the nanoparticle, which can be problematic. Nanocarriers often are designed to release their cargo in the body at specific sites for therapeutic purposes. Furthermore, various biochemical species in the body can break or displace chemical bonds on the nanoparticle surface [43,44] or disrupt the integrity of intact nanoparticles [45], leading to unintended leakage of cargo or detachment of labeled tags as shown schematically in Figure 3b [46]. For example, a dual radiolabeling to track gold nanoparticles in mice found different biodistribution profiles for the core and surface coating [47]. It is also important to note that addition of an exogenous tag or label can substantially alter nanoparticle behavior *in vivo* [48]. For example, Alamo et al. showed that ATTO488 and sulfo-Cy5 dye-labeled protein

Figure 4



Ex vivo methods of quantifying nanoparticle delivery. **(a)** Schematic of *ex vivo* tracking methods for nanoparticle-based drug delivery, including broadly categorized quantification methods of imaging, spectrometry, immunodetection, and sequencing. **(b)** Summary table for compatibility of *ex vivo* quantification methods for different nanocarriers and cargo.

nanoparticles accumulated in off-target organs more with reduced tumor targeting compared with unlabeled nanoparticles [49].

Ex vivo carrier and cargo tracking

In vivo nanoparticle delivery quantification offers the advantage of longitudinal tracking and best preserves the context of the entire biological system. Conversely, *ex vivo* quantification of nanoparticle delivery can provide greater resolution, sensitivity, and precision [50]. *Ex vivo* methods require organ and tissue biopsy or necropsy, and blood or bodily fluid collection following nanoparticle administration into an animal model of choice. Broadly categorized, various optical imaging, immunodetection, spectrometry, and sequencing techniques can be utilized to quantitatively assess the distribution and concentration of nanoparticle carriers and/or their cargo (as summarized in Figure 4).

All *in vivo* imaging techniques for nanoparticle tracking described previously are amenable for *ex vivo* imaging as well. Notably, *ex vivo* imaging can yield improved resolution and sensitivity since there are no movement artifacts and generally no constraint on imaging time [50]. There are also additional high-resolution imaging modalities that can be used for *ex vivo* nanoparticle quantification such as super-resolution microscopy [51], electron microscopy [52], and tissue clearing techniques to render the whole organs and animals transparent for 3D optical imaging [53,54].

Immunodetection and histochemistry techniques leverage the specific binding between antibodies and antigens, or affinity between stains and chemical moieties to detect molecules within cells and tissues [55]. For example, antibodies against different exosomal marker proteins, including TSG101, CD63, and HSP70, can be used in Western blot, flow cytometry, and immunofluorescence to track extracellular vesicles in cells and blood circulation [56]. In another example, Korangath et al. stained iron oxide nanoparticles using Prussian blue (Perl's reagent) to visualize their distribution in a mouse tumor in histology [57].

Spectrometric techniques are useful for measuring nanocarrier and cargo concentrations in tissues. Mass spectrometry, combined with different sample separation and introduction systems, can detect various analytes of interest. Inductively coupled plasma mass spectrometry (ICP-MS) can measure trace levels of multiple elements in biological fluids at the same time [58]. For nanocarrier tracking, Albanese et al. used ICP-MS to simultaneously quantify cell uptake of metallic and semiconductor nanoparticles, normalizing for cell mass by also measuring endogenous magnesium [59]. For cargo tracking, Song et al. used liquid chromatography–tandem mass spectrometry (LC–MS/MS) to quantitate the total versus released drug concentrations from polymeric nanoparticles in monkey plasma [60]. Mass spectrometric imaging additionally offers spatial nanocarrier and cargo concentration information, using techniques such as matrix-assisted laser desorption ionization or laser ablation to raster serially across the tissue surface to extract analytes and map ion images with nanometer and micrometer resolution [61].

Sequencing and related techniques are valuable tools for tracking and quantifying nucleic acid-based nanocarriers and cargo. Fluorescence in situ hybridization (FISH), and its many derived variants, uses fluorescent probes to label specific DNA or RNA sequences based on Watson–Crick base pairing for imaging [62]. For example, single-molecule FISH imaging can map the subcellular location of mRNA cargo delivered by lipid nanoparticles in fixed tissues [63]. Amplification methods can be additionally applied to enhance their limit of detection. Wang et al. developed origamiFISH, which uses hybridization chain reaction probes for up to 1000-fold signal amplification to map DNA nanostructures in tissues at picomolar concentrations [64]. Next-generation sequencing (NGS) sequences short DNA or RNA at high throughput, scalability, and speed [65]. Assigning DNA barcodes to different nanoparticle designs allows simultaneously testing of thousands of designs *in vitro* and *in vivo*, with NGS used to evaluate delivery at the organ and cellular levels by sequencing the barcodes [66].

Conclusion

In conclusion, quantifying nanoparticle delivery is complex due to the wide array of nanoparticle designs and the diverse methodologies used to track them. In this review, we discussed the three key approaches: functional readout, nanocarrier tracking, and cargo tracking. Functional readout evaluates therapeutic effects or reporter activity post nanoparticle administration. Nanocarrier tracking focuses on following the carrier itself within biological systems, while cargo tracking offers insights into the nanoparticles' contents or conjugated labels. These methods each have their respective merits and limitations. Researchers need to choose the appropriate methods or integrate multiple approaches to obtain a more accurate and comprehensive understanding of nanoparticle behavior. Moving forward, the establishment of standardized methodologies for quantifying nanoparticle delivery should be prioritized to enable effective comparisons of nanoparticle designs and formulations and accelerate their clinical translation.

CRediT authorship contribution statement

Conceptualization: WP, DEA, and MCC. Methodology: WP. Validation: WP, DEA, and MCC. Formal analysis: WP. Investigation: WP, DEA, and MCC. Resources: WP, DEA, and MCC. Data curation: WP, DEA, and MCC. Writing – original draft: WP, DEA, and MCC. Writing – review & editing: WP, DEA, and MCC. Visualization: WP, DEA, and MCC. Supervision: WP. Project administration: WP. Funding acquisition: WP and DEA. Both DEA and MCC contributed equally and have the right to list their name first in their CV. All authors contributed to the review and approved the submitted version.

Data Availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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- •• of outstanding interest

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