

RWD



White Paper on *In Vivo* Imaging Technology

Tumor Research · Drug Discovery · Immunology Research · Exosome Tracking

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Chapter 1 Background of Small Animal In Vivo Imaging

1.1 Common Modalities of Small Animal In Vivo Imaging

In vivo imaging acquires dynamic information at the physiological, pathological, and molecular levels in living animals through non-invasive or minimally invasive methods. Compared with traditional *ex vivo* experiments, *in vivo* imaging reduces individual variability, improves experimental reproducibility, and enables long-term longitudinal observation. Small animals (e.g., mice and rats) have become the primary models for biomedical research due to their high genetic tractability and relatively low cost. Continuous improvements in the sensitivity, resolution, and quantitative analysis capabilities of imaging technology have significantly advanced precision and translational medicine. With the advancement of optical imaging, nuclear medicine imaging, magnetic resonance imaging, and multimodal fusion, these technologies play a critical role in oncology, neuroscience, immunotherapy, and drug discovery. Currently, *in vivo* imaging for animals is mainly divided into the following five categories: optical imaging, radionuclide imaging, magnetic resonance imaging, computed tomography, and ultrasound imaging.

1.1.1 Visible Light Imaging

Currently, *in vivo* visible light imaging is mainly divided into bioluminescence imaging (BLI) and fluorescence imaging (FLI). The underlying principle of bioluminescence imaging involves integrating the luciferase gene into chromosomal DNA to express the luciferase. Upon administration of the exogenous substrate luciferin, the cells emit fluorescence, with the signal intensity linearly proportional to the number of labeled cells. In mammals, the luciferase gene is commonly used to label cells. Due to the absence of background signal interference from tissue autofluorescence, bioluminescence is widely used in animal model development. However, emission wavelengths vary among different luciferases; for instance, the commonly used firefly luciferase emits at approximately 550–570 nm, facilitating deeper tissue penetration. Fluorescence imaging utilizes fluorophores, such as green fluorescent protein (GFP) and red fluorescent protein (DsRed), to label nanomaterials or cells. These fluorophores are excited to a high-energy state and subsequently emit light for imaging. Currently, with the development of fluorescent dyes and fluorescent proteins, multiplexing labeling has

become widely applied in in vivo imaging. Since animal tissues exhibit autofluorescence, the selection of fluorescent dyes is critical, and requirements for instrumentation and algorithms are becoming more stringent.

1.1.2 Radionuclide Imaging

Radionuclide imaging is a molecular imaging technique based on radiotracers that achieves non-invasive visualization of physiological and biochemical processes in living organisms by detecting gamma rays or positrons released during radionuclide decay. This technique offers high sensitivity, functional imaging advantages, and quantitative analysis capabilities, making it widely used in clinical diagnostics and basic research. Currently, the primary radionuclide imaging techniques include single-photon emission computed tomography (SPECT) and positron emission tomography (PET). PET employs tracers labeled with positron-emitting nuclides (such as ^{18}F , ^{11}C). It utilizes the pair of gamma photons emitted in opposite directions when positrons produced during nuclide decay annihilate with surrounding electrons. Detectors monitor these photons in real time to determine the tracer's location, subsequently reconstructing tomographic images. Metabolically active tissues and organs exhibit increased uptake of ^{18}F -FDG; in tumor diagnosis, ^{18}F -FDG PET is typically used to localize tumors by detecting the hypermetabolic characteristics of tumor cells. SPECT utilizes single photons emitted by radionuclides (such as $^{99\text{m}}\text{Tc}$, ^{131}I), capturing information as the detector rotates around the body and reconstructing tomographic images through computer processing. It can be used for myocardial perfusion imaging, bone scintigraphy, etc. For example, in the diagnosis of myocardial ischemia, it can clearly visualize myocardial blood perfusion. Radionuclide imaging is often fused with structural imaging techniques such as CT to form integrated imaging systems like PET/CT and SPECT/CT. This fusion combines the functional metabolic information provided by radionuclide imaging with the detailed anatomical structure information provided by CT, thereby improving diagnostic accuracy and localization precision.

1.1.3 Magnetic Resonance Imaging

Magnetic Resonance Imaging (MRI) is a non-invasive medical imaging technology based on the principle of nuclear magnetic resonance (NMR), which utilizes strong magnetic fields and radiofrequency pulses to excite hydrogen atoms (^1H) in human tissues and reconstruct high-resolution anatomical images by detecting their relaxation signals. MRI

offers advantages such as lack of ionizing radiation, excellent soft tissue contrast, and multi-parametric imaging, and is widely used in clinical diagnosis and scientific research. By adjusting pulse sequence parameters (such as repetition time TR and echo time TE), MRI can generate images reflecting different tissue characteristics, enabling "multi-functional imaging" and providing multi-dimensional evidence for qualitative disease diagnosis. For example, in the diagnosis of spinal cord lesions, T1WI shows the degree of spinal cord compression, T2WI identifies edema or hemorrhage, and contrast-enhanced T1WI determines the presence of tumor enhancement; the combination of these three can effectively distinguish between intervertebral disc herniation and spinal cord glioma. Furthermore, MRI allows for axial, sagittal, coronal, and arbitrary oblique imaging without changing the patient's position, providing a comprehensive view of the spatial relationship between lesions and surrounding structures, making it particularly suitable for assessing complex anatomical regions. However, the sensitivity of MRI is relatively lower than that of PET/SPECT; therefore, MRI can form a complete diagnostic system by complementing other imaging technologies.

1.1.4 Computed Tomography

Computed tomography (CT) is a medical imaging technique based on X-ray technology that generates 3D images of internal body structures by using computer processing of multi-slice projection data. The underlying principle of CT is to utilize the penetrability of X-rays and the differential attenuation across various tissues to convert multi-angle projection data into tomographic images via computer algorithms. Specifically, X-rays are high-energy electromagnetic waves; when penetrating the human body, they undergo varying degrees of attenuation (absorption or scattering) depending on the density, atomic number, and thickness of the tissues. Micro-computed tomography (micro-CT) is a miniaturized version of clinical CT technology, designed specifically for experimental animals such as mice and rats. Compared to clinical CT, its spatial resolution can reach the micron level (1–50 μm). Small animals (especially rodents) are classic models for the study of bone development, degenerative diseases (such as osteoporosis), and hereditary bone diseases; due to its high sensitivity to bone structure, CT has become a core imaging tool in this field. For mouse models of rheumatoid arthritis, osteoarthritis, and similar conditions, CT allows for the dynamic observation of pathological changes such as joint space narrowing, subchondral bone sclerosis, and bone hyperplasia. For example, longitudinal CT scans of the knee joints in collagen-

induced arthritis (CIA) rat models can quantify the extent of periarticular bone erosion and assess the efficacy of anti-inflammatory drugs. Micro-CT also offers unique advantages in cardiovascular imaging; calcified lesions within atherosclerotic plaques are high-sensitivity targets for CT due to the high attenuation coefficient of calcified tissue. In ApoE knockout mice (a classic atherosclerosis model), CT can detect calcified plaques in the aortic root and carotid arteries, quantify calcification volume and density, and evaluate the effects of lipid-lowering drugs or genetic interventions on plaque progression.

1.1.5 Ultrasound Imaging

Ultrasound imaging is a non-invasive medical imaging technology based on the physical properties of ultrasound waves. It generates real-time anatomical or functional images by emitting high-frequency sound waves (ultrasound) into the human body or an organism and processing the received reflection, scattering, or transmission signals via a computer. The core principle of ultrasound imaging relies on the differences in propagation characteristics (such as speed of sound, attenuation coefficient, and reflection coefficient) of ultrasound waves across different tissues.

Ultrasound imaging can be categorized into several types based on imaging mode and function, each with a specific focus. For example, B-mode ultrasound is the most fundamental imaging mode; it displays the two-dimensional anatomical structure of tissues in grayscale, where brightness reflects the intensity of the reflected signal. Doppler ultrasound is typically used to detect blood flow velocity and direction, enabling the visualization of vascular distribution and hemodynamic status. Despite its wide range of applications, ultrasound still has limitations. Limited tissue penetration and interference from gas and bone often necessitate combining ultrasound with CT or MRI for multimodal imaging.

1.2 Current Technological Status and Research Trends

Before the concept of "small animal imaging" emerged, experimental animals could only be imaged using clinical instruments. However, preclinical research mostly uses rodent models (mice or rats), and in many cases, these models are too small to be accurately imaged by clinical imaging equipment. Therefore, the development of small animal in vivo imaging focuses on higher resolution, deeper penetration, multimodal integration,

and intelligent analysis, while emphasizing the reduction of animal distress and the enhancement of clinical translation potential. In recent years, single imaging modalities have often faced inherent drawbacks due to the limitations of their underlying principles, leading to incomplete information acquisition and restricted applications. These shortcomings have driven the development of multimodal imaging technology—integrating the advantages of different modalities (e.g., PET's functional metabolic information + CT's anatomical localization, or MRI's soft tissue contrast + photoacoustic molecular specificity) to obtain more comprehensive and precise research data. Small animal in vivo optical imaging primarily includes bioluminescence imaging (BLI) and fluorescence imaging (FLI), which are widely used in fields such as oncology, immunology, neuroscience, and drug development due to their advantages of high sensitivity, real-time dynamic monitoring, and low cost. Its core advantages lie in the following areas:

(1) High sensitivity, capable of detecting trace biological signals

BLI (bioluminescence imaging): Can detect <100 cells (e.g., tumor micrometastases) and is suitable for low-abundance targets (e.g., circulating tumor cells, bacterial infections).

FLI (fluorescence imaging): Near-infrared (NIR) probes can penetrate several millimeters of tissue, enhancing deep-tissue signal detection (e.g., tumor vascular imaging).

Real-time dynamic monitoring, reducing the number of experimental animals

Longitudinal tracking: Multiple imaging sessions of the same animal minimize individual variability (e.g., tumor growth, immunotherapy response).

Pharmacokinetics: Real-time observation of drug distribution (e.g., targeting evaluation of nano-drug delivery systems).

(2) Simple operation and lower cost

Compared with PET/MRI, optical imaging equipment is more cost-effective and does not require radioisotopes or strong magnetic fields.

Suitable for high-throughput drug screening (e.g., antitumor drug efficacy evaluation).

(3) High specificity at the molecular level

Reporter gene systems (e.g., luciferase coupled with specific promoters) can monitor gene expression (e.g., p53 activation).

Functional probes (e.g., calcium ion or ROS sensors) enable dynamic observation of live cells.

(4) High compatibility with multimodal imaging

It can be used in combination with CT/MRI/PET to integrate anatomical and functional information (e.g., BLI for tumor localization, MRI for structural background).

1.3 Common Experimental Challenges

1.3.1.1 Signal is too weak to detect

This is the most common issue; the expected strong signal is almost invisible on the image and indistinguishable from the background, leading to experimental failure.

Reason:

- (1) Insufficient cell/probe dosage: The number of injected fluorescent cells is too low or the probe concentration is too low.
- (2) Low expression level: The expression efficiency of reporter genes (e.g., luciferase) or fluorescent proteins (e.g., GFP, RFP) in target cells is low.
- (3) Penetration depth limitations: Visible light (400–700 nm) undergoes severe scattering and absorption within biological tissues, especially blue-green light, which can barely be transmitted from deep tissues.
- (4) Rapid probe/substrate metabolism: For example, the luciferase substrate luciferin is rapidly metabolized in vivo; improper injection timing may result in missing the peak signal.
- (5) Insufficient instrument sensitivity: The camera or optical system cannot effectively capture weak photon signals.

1.3.1.2 High background noise, poor signal-to-noise ratio

The image background is "noisy," with non-specific signals obscuring the target signal, making it difficult to distinguish and quantify.

Reason:

- (1) Non-specific binding: Particularly in fluorescence imaging, probes can non-specifically accumulate in the reticuloendothelial system, such as the liver and spleen, or bind to serum proteins, resulting in high background signal.

- (2) Autofluorescence: Experimental animal feed (especially chlorophyll-rich diets), intestinal contents, fur, and certain drug metabolites can emit intense autofluorescence under specific excitation light, with spectra that may overlap with the target signal.
- (3) Ambient light interference: Insufficiently dark rooms or improperly sealed instrument doors leading to light leakage.

1.3.1.3 Unstable signals and poor reproducibility

Significant signal intensity variation among individuals within the same group, or erratic fluctuations in the same animal across different time points, prevents statistical analysis or longitudinal comparison.

Reason:

- (1) Inconsistent probe/substrate injection: Differences in injection volume, rate, and site (intraperitoneal vs. intravenous injection) significantly affect biodistribution and signal kinetics.
- (2) Individual animal variability: Metabolic state, body weight, age, and hormonal levels can affect probe metabolism and distribution.
- (3) Inconsistent anesthetic depth: Anesthesia affects the animal's body temperature and blood circulation, thereby impacting the distribution of luciferin and the enzymatic reaction rate, leading to fluctuations in the bioluminescence signal.

1.3.2 Operational and Technical Issues

1.3.2.1 Animal preparation is cumbersome and prone to introducing errors

Challenges: Incomplete depilation leading to background fluorescence; excessive or insufficient anesthesia; prolonged operation time causing decreased body temperature (affecting signal).

Specific issues: Poor efficacy or skin irritation from commercial depilatory creams; unstable isoflurane anesthesia systems; low reproducibility of manual injections.

1.3.2.2 Difficulties in image registration and localization

Challenges: Especially in systems without 2D X-ray projection, it is impossible to precisely determine the anatomical location of the signal source (e.g., whether it originates from the intestine, uterus, or tumor).

Reason: Abdominal signals in mice may originate from the intestines, liver, kidneys, or tumors; these are difficult to distinguish without anatomical references.

1.3.2.3 High subjectivity in data analysis and quantification

Challenges: How to define the region of interest (ROI)? How to set the background threshold? Different individuals analyzing the same image may obtain significantly different quantitative results.

Reason: Lack of a standardized analysis workflow. When signal edges are blurred, the ROI size significantly affects the calculation of total radiant flux.

1.3.3 Biological and Technical Limitations

1.3.3.1 "Seeing a signal" is not equivalent to "seeing cells"

Challenges: Misinterpreting inflammation, necrosis, or non-specific probe retention as signals from tumor cells.

Reason: The imaging technology provides biochemical signals rather than cellular morphological information. Inflammatory regions surrounding tumors may also contain numerous macrophages that engulf probes or express reporter genes, resulting in false positives.

1.3.3.2 Inability to perform absolute quantification

Challenge: It is difficult to accurately determine the exact number of cells within a tumor. Signal intensity is affected by numerous factors (depth, blood supply, hypoxia, pH, etc.), allowing only for relative comparisons (e.g., treatment vs. control groups).

Chapter 2 Applications of In Vivo Imaging in Tumor Research

2.1 Common Tumor Models

2.1.1 Spontaneous Tumor Models

Spontaneous animal tumor models refer to tumors that develop naturally during an animal's lifespan without genetic manipulation or carcinogen exposure. Primary spontaneous animal tumor models currently include mammary, liver, and lymphosarcoma models.

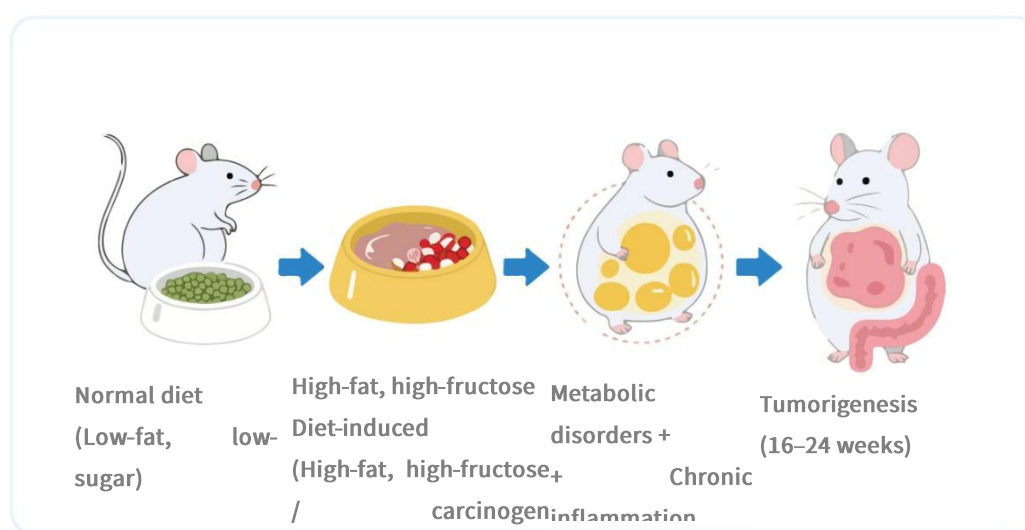


Figure 1 Schematic of spontaneous animal tumor model development

2.1.1.1 Spontaneous breast cancer mouse models

Inbred mouse strains are commonly used, with reported strains including C3H, A, and CBA/J; among them, C3H mice have a relatively high incidence of spontaneous breast cancer.

C3H/HeJ mice: The spontaneous incidence of breast tumors in female mice at 6–12 months of age is >85%. C3H/HeJ female mice were maintained until 6–12 months of age to observe spontaneous mammary tumor formation. The tumor type is primarily adenocarcinoma, which is similar to human breast cancer. Tumorigenesis is associated with mouse mammary tumor virus (MMTV) infection.

Spontaneous mammary tumors in C3H/HeJ mice are predominantly hormone-dependent (associated with estrogen and progesterone) and mimic human hormone

receptor-positive (HR+) breast cancer. Consequently, they are frequently used to elucidate the molecular mechanisms by which hormones regulate cell proliferation and differentiation during tumorigenesis, or to investigate the link between endocrine disorders (such as postmenopausal hormone fluctuations) and breast cancer risk. Additionally, these spontaneous mammary tumors frequently metastasize to the lungs and lymph nodes, making them suitable for tracking the complete metastatic cascade—from primary tumor detachment and intravasation to colonization of distant organs. They are also used to characterize the roles of metastasis-related molecules (e.g., integrins, chemokines) or to identify therapeutic targets for inhibiting metastasis. These models are also utilized to analyze phenotypic and functional shifts in tumor-infiltrating immune cells (e.g., T cells, macrophages, NK cells), such as the establishment of an immunosuppressive microenvironment, to explore immune escape mechanisms and evaluate the efficacy of immune checkpoint inhibitors (e.g., PD-1/PD-L1 antibodies) against spontaneous tumors. However, because C3H/HeJ mice are insensitive to lipopolysaccharide (LPS) due to a Tlr4 gene mutation, their immune response (e.g., inflammation-related pathways) may be affected; therefore, this characteristic must be considered when studying "inflammation-cancer" associations or immunotherapy to avoid biased results.

2.1.1.2 Spontaneous mouse models of liver tumors

Spontaneous liver tumors in mice usually originate from hepatocytes, and the development and progression of liver tumors generally begin with hepatocyte injury leading to inflammation. Persistent inflammatory stimulation, in turn, leads to the excessive deposition of extracellular matrix (ECM), forming a fibrotic scaffold. Finally, tumor cells acquire invasive and metastatic capabilities, manifested by vascular invasion, satellite nodule formation, and distant metastasis (such as lung metastasis). The following mouse strains are reported to be commonly used for spontaneous liver tumor modeling.

Table 1 Mouse Strains Commonly Used for Spontaneous Liver Tumor Modeling

Strain	Tumor incidence	Time to onset	Primary pathological types
C3H/HeJ	80–90%	12–18 months	Hepatocellular carcinoma
B6C3F1	30–50%	18–24 months	Adenoma/Carcinoma
PTEN+/-	60%	6–9 months	Combined hepatocellular-cholangiocarcinoma

Animals should be provided with ad libitum access to sterilized feed (AIN-93G formula). Additionally, models can be established through chemical or dietary induction.

Chemical induction methods typically include diethylnitrosamine (DEN) induction and aflatoxin (AFB1) induction. For diethylnitrosamine (DEN) induction, outbred rats weighing approximately 250 g or specific mouse strains of either sex can be used; animals should be sex-segregated and provided with standard chow and water. Administer 0.25% DEN aqueous solution by gavage at a dose of 10 mg/kg, once a week; for the remaining 5 days, provide 0.025% DEN aqueous solution in water bottles for ad libitum consumption. This can induce liver cancer after approximately 4 months of treatment. Alternatively, mice can be given 0.005% DEN in their drinking water ad libitum, which can induce liver cancer within 8 months. The aflatoxin B1 (AFB1) induction method involves mixing AFB1 into mouse feed at a dose of 0.001–0.015 ppm for 6 months, achieving a liver cancer induction rate of up to 80%. The dietary induction method is primarily based on a high-cholesterol diet. Select a high-fat/high-cholesterol diet (e.g., containing 43.7% fat, 36.6% carbohydrates, 19.7% protein, and 0.203% cholesterol). Eight-week-old male C57BL/6 wild-type littermates are fed a high-cholesterol diet for 14 months. Research indicates that mice fed this diet exhibited elevated serum AFP levels at 10 months, developed liver tumors at 14 months, and histological examination of liver sections confirmed hepatocellular carcinoma.

2.1.2 Transplantable tumor models

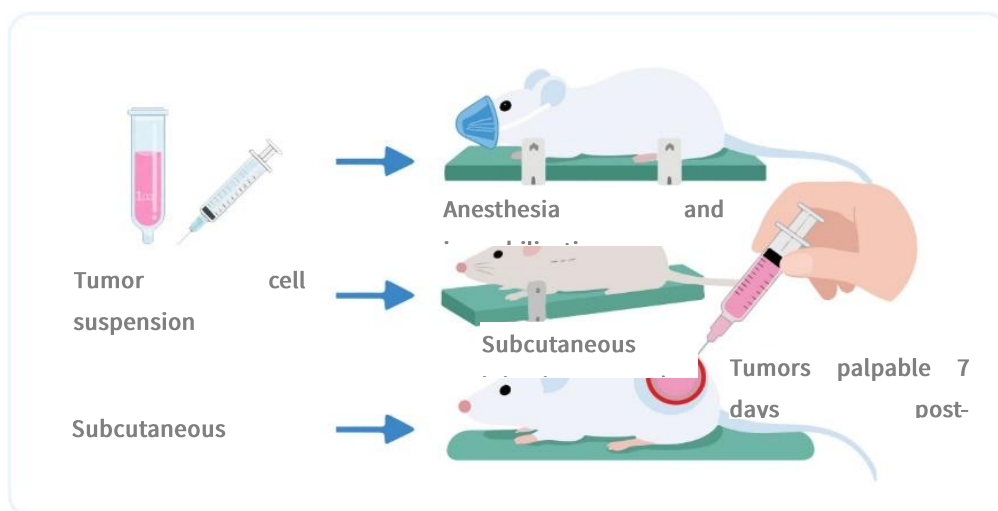


Figure 2 Schematic of transplantable animal tumor model establishment

A transplantable tumor model is one in which tumor cells, tissues, or organs are transplanted into experimental animals to induce tumor growth. Due to their short establishment cycle, high reproducibility, and ability to effectively simulate tumor growth and metastasis, these models are widely used in cancer research, playing a crucial role in drug screening, efficacy evaluation, and the study of metastatic mechanisms. Based on the source of the transplanted tumor and the immune status of the recipient animal, transplantable tumor models can be classified into the following categories:

2.1.2.1 Allograft models

These models are established by transplanting tumor tissues or cells into animals of the same strain. Because the donor and recipient share the same genetic background, the immune system typically does not mount a strong rejection response, facilitating tumor growth.

Advantages: Good stability, relatively consistent growth characteristics, high reproducibility, and effective simulation of tumor growth within a syngeneic host.

Applications: Frequently used to evaluate the efficacy of antitumor drugs, study tumor growth patterns, and investigate mechanisms of invasion and metastasis. For example, transplanting mouse H22 hepatoma cells intraperitoneally or subcutaneously into mice of the same strain creates a syngeneic hepatoma model for drug screening.

2.1.2.2 Xenograft models

These models are established by transplanting human tumor tissues or cells into immunodeficient animals. Due to species differences between human and animal cells, the immune system of immunocompetent animals will reject the graft. Therefore, immunodeficient animals must be used as recipients, such as nude mice (athymic, T-cell deficient) and SCID mice (T- and B-cell deficient).

Advantages: These models directly reflect the biological characteristics of human tumors, such as morphology, growth rate, and drug sensitivity, providing a more clinically relevant model for preclinical research.

Applications: Widely used in studying tumor pathogenesis, drug discovery, and evaluating personalized treatment strategies. For example, subcutaneous inoculation of human lung cancer A549 cells into nude mice can establish a human lung cancer xenograft model for testing novel therapeutics.

2.1.2.3 Orthotopic Transplantation Models

Tumor cells or tissues are transplanted into the corresponding primary organ or tissue of the recipient animal. For example, transplanting hepatocellular carcinoma cells into the mouse liver, or transplanting breast cancer cells into the mouse mammary fat pad, etc.

Advantages: The microenvironment of tumor growth is more similar to that of the primary tumor, which better simulates biological behaviors such as tumor invasion and metastasis, making it an ideal model for studying organ-specific metastasis and the efficacy of local treatment.

Applications: Commonly used to study the growth mechanisms of tumors in specific organs and to evaluate the efficacy of local treatments such as surgery and radiotherapy. Commonly used methods for transplantable tumor models mainly include cell suspension inoculation and tissue block transplantation.

(1) Cell suspension inoculation

Preparation of tumor cells: Select tumor cells in the logarithmic growth phase, collect them via centrifugation after trypsinization, and adjust the cell concentration to an appropriate level (usually 1×10^6 - 1×10^7 cells/mL) using normal saline or serum-free medium.

Inoculation procedure: Use a syringe to inoculate the cell suspension into specific sites of the recipient animal, such as subcutaneously, intraperitoneally, intravenously, or orthotopically within organs. For subcutaneous inoculation, the back or axilla is generally chosen; intraperitoneal inoculation can form ascites tumors; intravenous inoculation is often used to study tumor metastasis; and intra-organ inoculation is used to establish orthotopic transplant models.

(2) Tissue block transplantation

Tumor tissue preparation: Select fresh tumor tissue (either syngeneic or human specimens) and cut into uniform blocks (typically 1–2 mm³) under aseptic conditions.

Inoculation procedure: Make a small incision at the inoculation site of the recipient animal, implant the tissue block, and then suture the incision. For orthotopic transplantation, the tissue block must be precisely implanted into the corresponding organ.

2.2 Workflow for Establishing Visualized Tumor Models

2.2.1 Construction of Luciferase Reporter Gene System

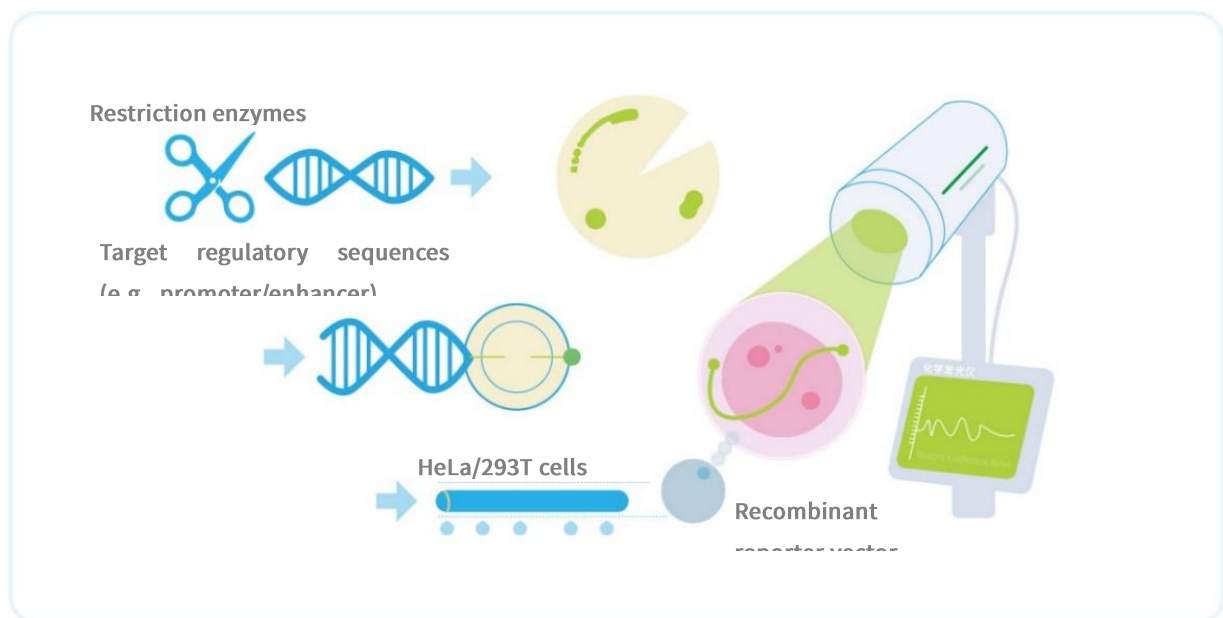


Figure 3 Schematic of Transplantable Animal Tumor Model Establishment

2.2.1.1 System components

Reporter gene vector: Contains a luciferase gene (e.g., Luc2-optimized firefly luciferase) and a multiple cloning site (MCS) for inserting the promoter/enhancer sequences under study. Commonly used vectors: pGL3-Basic (promoterless), pGL4 series (lower background, higher sensitivity).

Internal control vector: Used to normalize for transfection efficiency variations, typically utilizing a different luciferase (e.g., Renilla luciferase, such as pRL-TK or pGL4.74). Other internal controls (such as β -galactosidase or fluorescent proteins) can also be used.

Substrates: Firefly luciferase: D-luciferin (requires ATP, O₂, Mg²⁺). Renilla luciferase: Coelenterazine.

2.2.1.2 Construction steps

(1) Target sequence cloning

Promoter/enhancer fragment acquisition: Amplify the target sequence from genomic DNA via PCR or obtain it through chemical synthesis (typically 500–2000 bp).

Vector construction: Insert the fragment into the multiple cloning site (MCS) of the reporter vector (e.g., HindIII/XhoI sites of pGL3-Basic).

Validation: Confirm insertion orientation and sequence accuracy via sequencing.

(2) Reporter vector optimization

Removal of cryptic regulatory elements: Ensure the vector backbone does not contain transcription factor binding sites that interfere with the target promoter.

Addition of polyadenylation signal (polyA): Enhance mRNA stability (e.g., SV40 polyA).

(3) Co-transfection system design

Experimental group: Reporter vector (e.g., pGL3-Promoter) + internal control vector (e.g., pRL-TK).

Control group: Empty vector (pGL3-Basic) as negative control; positive control (e.g., pGL3-Control with SV40 promoter).

(4) Cell transfection

Suitable cell lines: HEK293 (high transfection efficiency), HepG2, or other relevant cell types.

Transfection methods: Liposome (e.g., Lipofectamine 3000), electroporation, or viral transduction.

Transfection ratio: The ratio of reporter vector to internal control vector is typically 10:1 to 50:1 (e.g., 1 µg pGL3 : 0.1 µg pRL-TK).

(5) Luciferase activity assay

Cell lysis: 24–48 hours after transfection, lyse the cells using lysis buffer.

Dual-luciferase assay (using the Promega Dual-Luciferase system as an example):

Add the firefly luciferase substrate to detect firefly luciferase activity (the signal persists for approximately 10 seconds).

Add the reagent to quench firefly luciferase and activate Renilla luciferase to detect internal control activity.

(6) Data analysis

Calculate relative activity: Firefly RLU / Renilla RLU to normalize for transfection efficiency.

Statistical analysis: Use t-test or ANOVA to compare differences between experimental and control groups.

2.2.2 Construction of Fluorescent Protein Reporter Gene Systems (using green fluorescent protein GFP as an example)

2.2.2.1 Construction of the GFP reporter gene system

The core goal of construction is to link the GFP gene with the "target sequence" (regulatory element or target gene) via a vector to form an expressible recombinant system. The steps are as follows:

(1) System design: Define the objective

Determine the system type according to research requirements; common types include:

1) Promoter/enhancer activity reporter system: The GFP gene is placed downstream of the target promoter/enhancer; GFP fluorescence intensity reflects the activity of the regulatory element (e.g., detecting promoter activation under hypoxic conditions).

- 2) Gene expression tracking system: GFP is fused with the target gene (e.g., target gene-GFP) to visualize the spatiotemporal expression pattern or subcellular localization of the target protein (e.g., tracking the dynamic distribution of cytoskeletal proteins).
- 3) Signaling pathway reporter system: Link the GFP gene to a pathway-specific downstream promoter (e.g., NF- κ B response element) to reflect pathway activation (e.g., inflammatory pathways) via fluorescence.

(2) Vector backbone selection

It is necessary to select an appropriate plasmid vector as the backbone; key elements include:

- 1) Origin of replication: Ensures stable replication of the vector in host cells (e.g., pcDNA series for mammalian cells, pET series for prokaryotes).
- 2) Selection marker: Used to select cells that have successfully taken up the vector (e.g., antibiotic resistance genes such as neo or amp, or a fluorescent marker like mCherry).
- 3) Promoter: For promoter reporter systems, the vector's endogenous promoter must be removed, leaving only the multiple cloning site (MCS) for target promoter insertion; for fusion protein systems, a strong promoter (e.g., CMV, EF1 α) is required to drive expression.
- 4) Multiple cloning site (MCS): Must contain restriction sites matching the target fragment without disrupting the open reading frame (ORF) of GFP.

(3) Preparation of key fragments

The following core fragments must be obtained via PCR amplification or gene synthesis:

- 1) Target sequence: For a promoter reporter system, clone the target promoter/enhancer sequence (typically 500–2000 bp in length, must include the core regulatory region); for a fusion protein system, clone the full-length target gene (ensure it lacks a stop codon to avoid preventing GFP expression).
- 2) GFP gene: Commonly used optimized variants (e.g., EGFP for stronger fluorescence; mGFP for higher stability) must have an intact ORF that is in-frame with the target sequence (especially critical for fusion protein systems).

(4) Recombinant Vector Construction

The target sequence and GFP gene are ligated into the vector backbone using molecular cloning techniques. Common methods include:

Restriction digestion and ligation:

- a. Digest the vector backbone, target sequence, and GFP gene with the same restriction enzymes (e.g., EcoRI, XhoI). In fusion systems, the target gene and GFP should be joined via a linker to prevent steric hindrance.
- b. Ligate the digested fragments using T4 DNA ligase to form the recombinant vector.

Homologous recombination:

Homology arms are introduced via primer design, allowing recombinases (e.g., In-Fusion, Gibson Assembly) to directly ligate fragments into the vector. This method bypasses restriction digestion, offers higher efficiency, and is particularly suitable for complex fragment assembly.

(5) Initial vector verification

After recombinant vector construction, verification is required to prevent subsequent experimental errors:

Restriction digestion analysis: Digest the recombinant vector with the restriction enzymes used during construction and use agarose gel electrophoresis to verify if fragment sizes match expectations (e.g., a promoter-GFP vector should yield fragments corresponding to the vector backbone, promoter, and GFP).

Sequencing verification: Perform Sanger sequencing on the insert (target sequence + GFP) to confirm the sequence is mutation-free and correctly oriented (specifically, the promoter must be in the 5'→3' direction to drive GFP expression).

2.2.2.2 Verification of the GFP reporter gene system

Following construction, the system's effectiveness must be validated through multi-dimensional assays, including expression, functional, and specificity verification.

(1) Expression verification: Validate correct GFP expression

The core objective is to detect GFP expression at the transcriptional and translational levels to rule out vector construction errors or expression barriers.

Fluorescence microscopy: Transfect/transform the recombinant vector into the target host (e.g., mammalian cells, bacteria, plant cells), and after culture, observe green fluorescence using fluorescence microscopy (excitation wavelength 488 nm, emission wavelength 507 nm). The presence of fluorescence indicates successful

GFP expression; its absence necessitates troubleshooting the reading frame, promoter activity, or transfection efficiency.

Flow cytometry quantification: Perform flow cytometric analysis on transfected cells to determine the percentage of fluorescence-positive cells (reflecting transfection efficiency) and mean fluorescence intensity (reflecting expression levels).

Western blot analysis: Extract total protein and perform immunoblotting with a GFP-specific antibody; confirm correct protein synthesis by band size (e.g., ~27 kDa for EGFP) to rule out truncation or mistranslation.

RT-PCR/qPCR validation: Extract total RNA, perform reverse transcription, and amplify using GFP specific primers to detect mRNA levels, validating whether the expression impairment originates at the transcription stage (e.g., promoter failure).

(2) Functional validation: Verifying the biological significance of the system

Based on the system design objectives, verify whether it reflects the target biological process:

Promoter/enhancer activity validation: Measure changes in fluorescence intensity under various conditions (e.g., different cell types, hormone treatment, drug stimulation). For example, when studying a hypoxia-responsive promoter (e.g., HIF-1 α target promoter), compare fluorescence intensity under normoxic and hypoxic conditions to confirm signal enhancement (reflecting promoter activation).

Fusion protein localization validation: Observe the subcellular localization of GFP fluorescence via confocal microscopy and perform colocalization with known markers (e.g., DAPI for the nucleus, MitoTracker for mitochondria) to confirm whether the fusion protein is expressed at the expected site (e.g., whether the "target gene-GFP" localizes to the cell membrane).

Signaling pathway activation validation: Treat cells with pathway-specific agonists (e.g., activating the NF- κ B pathway with LPS), detect whether GFP fluorescence intensity increases with pathway activation, and use qPCR to measure the expression of downstream endogenous genes to verify the consistency between the reporter system and the endogenous pathway.

(3) Specificity Validation: Excluding Confounding Factors

Ensure the specificity of the fluorescence signal through control experiments:

Negative control:

Transfect cells with an empty vector (no GFP) to exclude cellular autofluorescence or interference from the vector backbone;

Transfect with a "target sequence - mutant GFP" vector (e.g., a non-fluorescent GFP mutant) to exclude non-specific signals.

Positive control: Transfect with a known high-expression GFP vector (e.g., pEGFP-N1) to ensure the experimental system (transfection, detection) is effective.

Stability Validation:

Assess GFP expression stability during long-term culture (e.g., >10 passages) or in animal models (e.g., transgenic mice) to rule out gene silencing or vector loss.

2.2.2.3 Common problems and optimization strategies

No fluorescence or weak fluorescence:

Vector issues: Confirm the reading frame and promoter orientation via sequencing; switch to a strong promoter (e.g., CMV) or add an enhancer (e.g., SV40 enhancer).

Expression issues: Optimize transfection efficiency (e.g., adjust liposome ratios); use brighter GFP variants (e.g., mNeonGreen, which is 3x brighter than EGFP).

Non-specific fluorescence:

Reduce excitation intensity or shorten exposure time, or use DsRed (e.g., mCherry) as internal controls to exclude background interference.

Fusion protein dysfunction:

Adjust linker sequences (e.g., flexible GGGGS linkers) to prevent steric hindrance between GFP and the target protein; alternatively, test both N-terminal and C-terminal fusions (GFP-Target vs. Target-GFP) to identify the functional construct.

2.3 Tumor Imaging Data Analysis and Quantitative Methods

Luciferase kinetic curves (e.g., firefly luciferase)

Due to the rapid biodistribution of firefly luciferase in vivo and the tissue dependency of its kinetic characteristics, researchers typically need to establish a luciferase kinetic curve for the animal model before conducting formal experiments to determine the optimal time point for image acquisition following the injection of the luciferase substrate.

2.3.1 Preparation

2.3.1.1 Animal Model Selection

Transgenic animals stably expressing luciferase or model animals transfected/inoculated with a luciferase reporter vector (e.g., tumor-bearing mice with luciferase-expressing tumor cells) must be used. Ensure that the luciferase expression in the model is stable and biologically relevant (e.g., targeted expression in specific tissues/organs).

2.3.1.2 Reagents and Instruments

Substrate: D-luciferin sodium salt (dissolved in sterile PBS or saline, typically at 15–150 mg/mL, prepared fresh before use);

Instruments: In vivo imaging system (RWD MOIS HT series) and gas anesthesia system (e.g., isoflurane);

2.3.2 Experimental Procedure

2.3.2.1 Animal Pretreatment

Weigh the animals and calculate the injection dose of D-luciferin sodium salt based on body weight (common dose: 100 μ L per mouse at a concentration of 15 mg/mL, i.e., 150 mg/kg; adjust proportionally for rats).

2.3.2.2 Injection and Imaging Time-Point Design

Injection route: Select based on model characteristics; common routes include intraperitoneal injection (slower absorption, longer signal duration) or intravenous injection (rapid absorption, earlier signal peak).

Time-point setting: Cover the interval from seconds to hours post-injection, accounting for signal rise, plateau, and decay phases.

For example:

Intravenous injection: 0.5, 1, 2, 5, 10, 15, 20, 30, 45, 60 minutes;

Intraperitoneal injection: 5, 10, 15, 20, 30, 45, 60 minutes.

(Dense sampling is recommended in the early phase, with extended intervals later, to capture critical inflection points.)

2.3.2.3 In Vivo Imaging Procedure

Place the animal in the anesthesia induction chamber and anesthetize with isoflurane (2–3% for induction, 1–2% for maintenance), then transfer to the heated stage (37 °C) of the imaging chamber;

At preset time points, inject D-luciferin sodium salt solution via the intraperitoneal or intravenous route (ensure uniform injection speed to avoid leakage);

Acquire bioluminescence images at each time point: set the exposure time (typically 1–60 seconds, adjusted according to signal intensity) and the excitation/emission filters (a 560 nm emission filter is commonly used for luciferase), and record the radiance (expressed in p/s/cm²/sr).

2.3.2.4 Data Acquisition and Normalization

For each imaging session, define a region of interest (ROI) in the same area (e.g., tumor site, specific organ) to quantify the signal intensity; record the body weight, injection dose, and anesthesia status of each animal to minimize the impact of individual variation; use at least 3 animals per group and calculate the mean to improve reliability. The trend of the firefly luciferase kinetic curve is generally as follows:

(1) Rapid rise phase (initiation phase)

In the initial stage of the reaction, bioluminescence intensity rises rapidly to a peak as the enzyme binds to the substrate. The reaction rate during this phase is influenced by substrate concentration, enzyme concentration, and reaction initiation efficiency; the peak height reflects the initial reaction activity.

(2) Linear decay phase (steady-state phase)

After reaching the peak, bioluminescence intensity declines slowly and linearly over time. During this phase, the enzyme and substrate are in dynamic equilibrium, and the reaction rate is relatively stable, making it the critical interval for quantitative analysis (the slope of this phase is typically used to calculate enzyme activity).

(3) Plateau decay phase (substrate depletion phase)

As the substrate is gradually depleted, the reaction rate drops sharply, bioluminescence intensity approaches background levels, and the curve enters a slow decay phase.

2.4 Application

2.4.1 Visualization of an Orthotopic Glioma Model

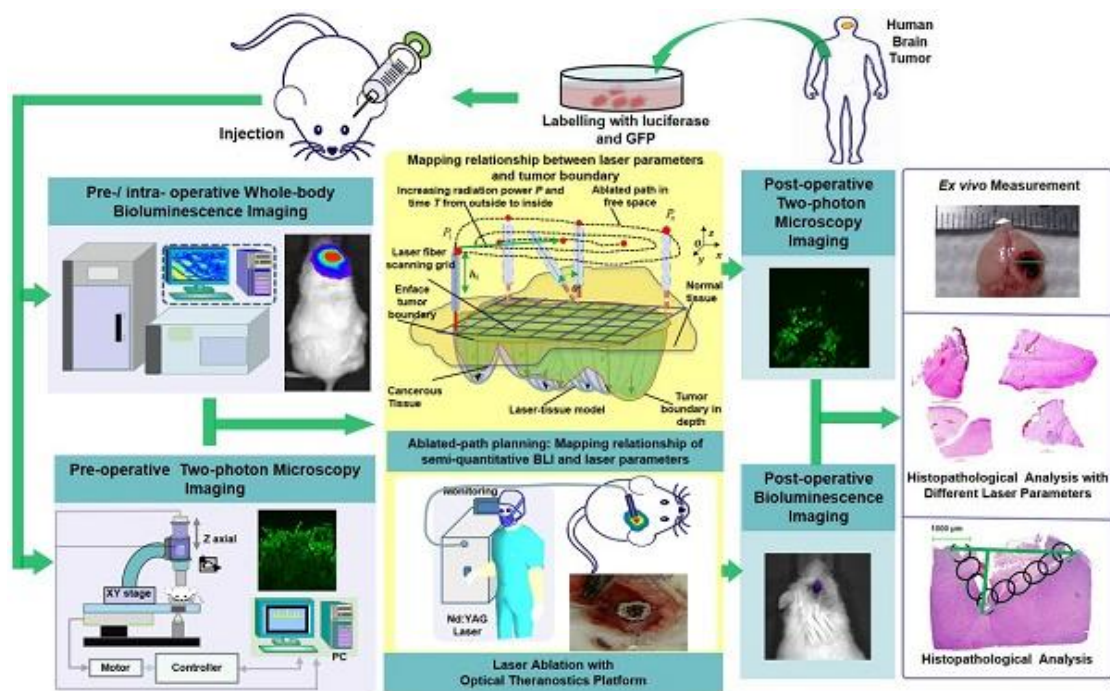


Figure 4 Schematic of Intraoperative Resection of Orthotopic Glioma

In this article, by imaging GBM cells expressing the luciferase reporter gene, the system can clearly visualize the extent and size of the tumor. The primary reason for GBM recurrence is the inability to completely resect infiltrative tumor cells during surgery; however, under BLI guidance, the precision of laser ablation reaches sub-millimeter levels, and the tumor ablation rate exceeds 99%. The platform successfully and clearly delineates the GBM margin, enabling precise resection. This study established a mapping relationship between BLI signal intensity (tumor burden) and laser ablation parameters, enabling quantitative and personalized surgical path planning to ensure that the laser covers all tumor regions. This study successfully demonstrated that a multimodal optical imaging platform, integrating BLI and TPM, can guide laser ablation therapy with extreme precision, achieving precise, minimally invasive, and efficient treatment of GBM. It provides a new paradigm for precision neurosurgery—integrating multiple real-time imaging techniques into a single therapeutic platform, allowing surgeons to "see" invisible tumor margins and perform precise resection^[1].

2.4.2 Establishment and Treatment of Bone Metastatic Breast Cancer Models

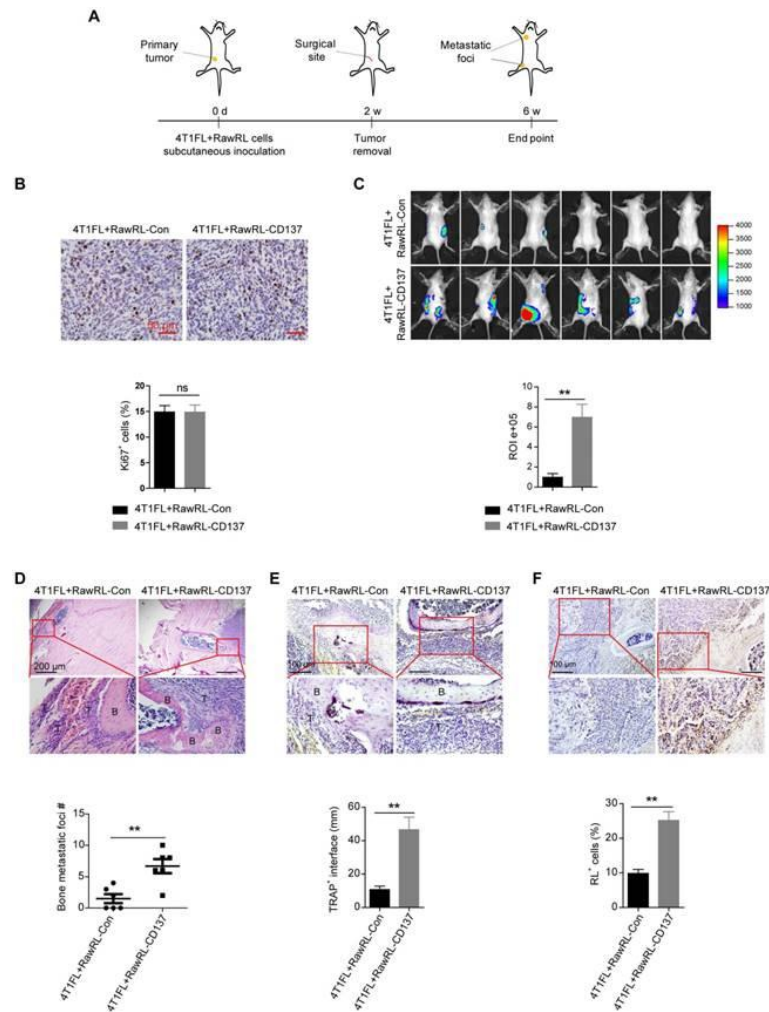


Figure 5 Therapeutic Effect of Nanoparticles on Bone Metastatic Breast Cancer

Bone is one of the most common sites of breast cancer metastasis, and CD137 (4-1BB) is a member of the tumor necrosis factor (TNF) receptor superfamily, primarily expressed in activated leukocytes. Existing studies have confirmed the role of the CD137-CD137L bidirectional signaling pathway in RANKL-mediated osteoclastogenesis. However, the role of CD137 in breast cancer bone metastasis remains to be further investigated. By establishing a spontaneous bone metastasis mouse model, the function of CD137 in bone metastasis in vivo was examined using bioluminescence imaging, immunohistochemistry, and histological examination. Prior research found that CD137 promotes the migration of monocytes/macrophages to the tumor microenvironment by upregulating *fr1* expression. It also promotes the differentiation of monocytes/macrophages into osteoclasts, providing a favorable microenvironment for the colonization and growth of breast cancer cells in bone. On this basis, a novel F4/80-

targeted nanoliposome encapsulating an anti-CD137 blocking antibody (NP- α CD137 Ab-F4/80) was synthesized. The nanoparticle exhibits high inhibitory effects on both bone and lung metastasis of 4T1 breast cancer cells in vivo. Furthermore, it enhances the therapeutic efficacy of Fra1 inhibitors against tumor metastasis^[2].

Notes: During the establishment of orthotopic metastatic tumor models, metastatic foci are often difficult to detect in the early stages due to the relatively low number of tumor cells. Therefore, to ensure successful model establishment, it is recommended to use high-sensitivity in vivo imaging instruments.

Chapter 3 Applications of In Vivo Imaging in Drug Development

3.1 Construction of Fluorescent Drug Delivery Systems

A fluorescent drug delivery system is an intelligent nanoplatform that integrates fluorescence imaging capabilities with drug delivery functions. By combining fluorescent labels (or fluorescence-responsive elements) with drug vehicles, these systems enable real-time visualization and tracking of the drug delivery process, precise localization at target sites, and dynamic monitoring of therapeutic effects, representing a typical application of the "theranostics" concept in pharmaceuticals. Its core advantage lies in overcoming the "black-box" limitations of traditional drug delivery, providing intuitive feedback on the in vivo distribution, release kinetics, and lesion targeting efficiency of drugs through fluorescence signals, which serves as a critical basis for optimizing drug delivery system design and improving therapeutic precision.

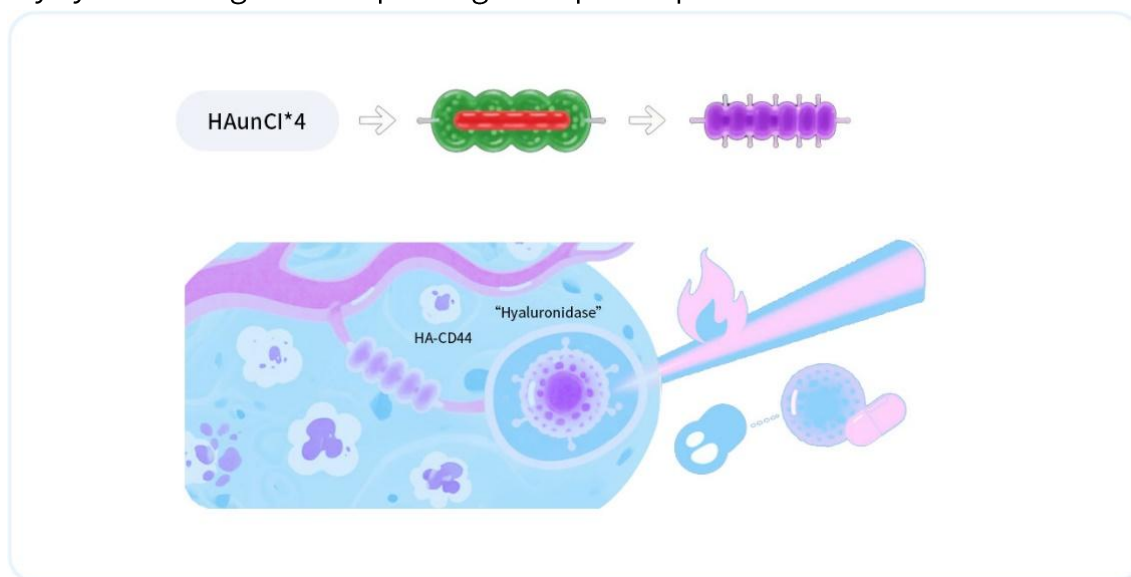


Figure 6 Schematic of the construction of a fluorescent drug delivery system

3.1.1 Core Components of the System

Fluorescent drug delivery systems typically consist of three key components that work synergistically to achieve "delivery-imaging-therapy" functions.

3.1.1.1 Drug Carrier (Core Scaffold)

The carrier is responsible for drug loading and delivery and must possess good biocompatibility, targeting ability, and controlled-release properties. Common types include:

Polymeric nanoparticles: Such as polyethylene glycol (PEG)-modified poly(lactic-co-glycolic acid) (PLGA) and chitosan; these can load hydrophobic drugs via hydrophobic interactions or covalent bonds and are easily modified with fluorophores.

Liposomes: Vesicular structures composed of a lipid bilayer, capable of encapsulating water-soluble drugs (in the internal aqueous phase) or lipid-soluble drugs (in the lipid bilayer), with surfaces that can be modified with fluorescent dyes (e.g., Cy5.5) and targeting ligands (e.g., RGD peptides).

Inorganic nanocarriers: Such as mesoporous silica nanoparticles (MSNs), quantum dots (QDs), and gold nanoclusters, which combine high drug-loading capacity with fluorescent properties (some are inherently luminescent, requiring no additional labeling).

Biomimetic carriers: Such as cell membrane-coated nanoparticles ("nanocamouflage"), which reduce immune clearance while enabling tracking via membrane surface fluorescent labeling.

3.1.1.2 Fluorescent Labels / Responsive Elements (Imaging Units)

Responsible for providing fluorescence signals to enable visualization of drug delivery. Selection must satisfy: high photostability, low biological toxicity, and an emission wavelength suitable for in vivo imaging (typically in the near-infrared region, 700–1000 nm, providing greater penetration depth and minimal tissue absorption). Common types include:

Organic fluorescent dyes: such as Cy3, Cy5.5, ICG (indocyanine green), have relatively low toxicity and are easily conjugated with carriers, but they suffer from rapid photobleaching (limiting long-term tracking).

Quantum dots (QDs): such as CdSe/ZnS quantum dots, possess high fluorescence quantum yield, strong stability, and tunable emission wavelengths (via size control). However, the presence of heavy metals (e.g., Cd²⁺) may cause long-term toxicity, necessitating surface modification (e.g., PEGylation) to improve safety.

Fluorescent proteins: such as GFP and mCherry, can be fused with carrier proteins via genetic engineering. They offer excellent biocompatibility but have relatively weak

fluorescence intensity and are primarily used for cell-level tracking due to insufficient penetration for in vivo imaging.

Stimuli-responsive fluorescent probes: such as pH-sensitive fluorescent molecules (fluorescence enhancement in the acidic tumor microenvironment) and enzyme-responsive probes (fluorescence recovery upon drug release), can simultaneously indicate the drug release status ("release-imaging" linkage).

3.1.1.3 Targeting Units (optional, for enhanced precision)

By conjugating targeting ligands (such as antibodies, peptides, and aptamers), the system's specific recognition of lesion tissues (e.g., tumors, inflammatory sites) is improved, reducing off-target effects. For example:

Tumor targeting: RGD peptides (which recognize tumor cell integrin $\alpha\beta_3$) and folate (which targets tumors with high folate receptor expression);

Inflammation targeting: Mannose (which recognizes the mannose receptor on the surface of macrophages).

3.1.2 Operating Principles and Typical Processes

The core of fluorescent drug delivery systems lies in the "correlation mapping" between fluorescence signals and drug delivery behavior, enabling real-time tracking from administration to therapeutic outcome. The typical process is as follows:

System construction: Drugs (such as chemotherapeutic or genetic drugs) are encapsulated or conjugated within a carrier, while fluorescent labels are introduced via covalent bonding, physical adsorption, or embedding, with targeting ligands modified as necessary.

Administration and in vivo transport: Following administration via intravenous or local injection, the system circulates, and the targeting units recognize and accumulate at lesion cell surface receptors (passive targeting relies on the EPR effect, while active targeting relies on ligand-receptor binding).

Fluorescence imaging tracking: Excitation of fluorescent labels via an in vivo imaging system (RWD MOIS HT series) allows for real-time monitoring of: drug distribution in the body (e.g., whether it accumulates in tumors rather than normal organs);

time taken to reach the target site (to optimize dosing intervals); changes in fluorescence intensity (indirectly reflecting drug concentration, e.g., signal enhancement suggests accumulation, while signal attenuation may accompany drug release).

Drug release and treatment: Triggered by the lesion microenvironment (e.g., low pH, highly reductive environment, specific enzymes), the carrier disintegrates to release the drug and exert its therapeutic effect; some systems can directly indicate release through changes in the fluorescence signal (e.g., fluorescence "turn-on" or "quenching" upon stimulation).

Therapeutic feedback: Continuous monitoring of changes in lesion size via fluorescence signals (e.g., tumor shrinkage accompanied by a decrease in the fluorescent area) allows for timely evaluation of treatment efficacy and adjustment of protocols.

3.2 Modification of Fluorescent Dyes

3.2.1 Chemical Structure Modification

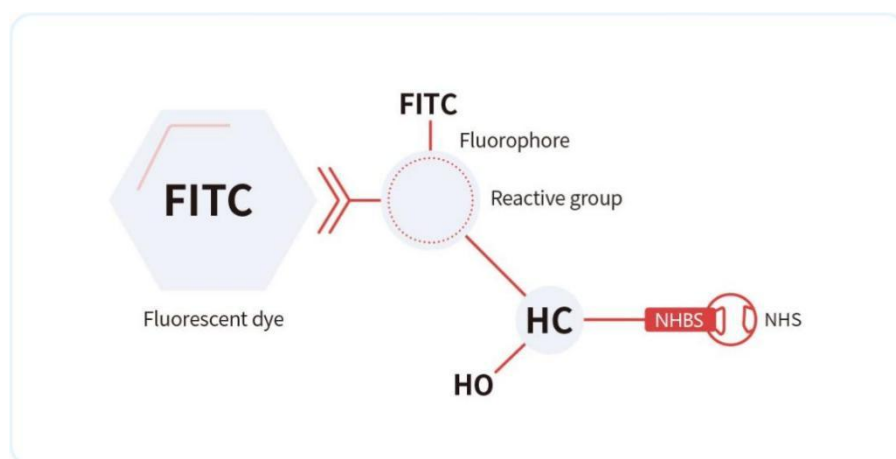


Figure 7 Schematic of Fluorescent Dye Construction

3.2.1.1 Introduction/Modification of Chromophores:

Extending the conjugated system (e.g., by adding benzene rings or polyene chains) to redshift the emission wavelength.

Example: Extension of cyanine dyes (Cy5)

- (1) Activation of starting materials: Reflux the indole derivative with iodoethane in acetonitrile to generate the quaternary ammonium salt intermediate.
- (2) Condensation reaction: Under basic conditions (e.g., triethylamine), the quaternary ammonium salt is condensed with glutaraldehyde to extend the conjugated chain.
- (3) Purification: The product is purified via silica gel column chromatography (eluent: dichloromethane/methanol).

Effect: The conjugated chain is extended from Cy3 (3 carbons) to Cy5 (5 carbons), and the emission wavelength is redshifted from ~550 nm to ~650 nm.

Introduce push-pull electron groups (e.g., -NH_2 , -NO_2) to modulate the fluorescence quantum yield.

3.2.1.2 Modification of the Fluorophore Core:

For instance, replacing the oxygen atom in rhodamine with silicon (Si-rhodamine) enhances photostability.

- (1) Construction of the xanthene skeleton: 2-bromobenzoic acid and resorcinol are condensed in molten ZnCl_2 to generate the oxarhodamine precursor.
- (2) Replacement of oxygen atom with silicon: The precursor reacts with SiCl_4 at high temperature, where the silicon atom replaces the oxygen bridge to form the SiR core.
- (3) Amine modification: The silicon core undergoes condensation with amines (such as N,N-diethylamine) to generate the final dye.

Effect: Replacement of the Si-O bond with a Si-C bond significantly reduces the photobleaching rate.

Fluorination (e.g., BODIPY fluorination) improves brightness and photostability.

- (1) BODIPY core synthesis: Pyrrole and aldehydes undergo condensation catalyzed by $\text{BF}_3 \cdot \text{Et}_2\text{O}$ to generate the BODIPY core.
- (2) Electrophilic fluorination: Fluorine atoms are introduced at the α -position (2,6-position) using Selectfluor[®] (a fluorinating reagent).
- (3) Purification: The fluorinated products are separated via preparative HPLC.

Effect: Fluorine atoms suppress molecular vibration and reduce non-radiative decay, increasing the quantum yield to >90%.

3.2.2 Functional Group Derivatization

3.2.2.1 Carboxyl Group (-COOH) Activation: Amide bond formation via EDC/NHS condensation with amino groups (-NH₂), used for bioconjugation (e.g., antibody labeling).

(1) Reagent preparation

EDC solution: Dissolve in water or anhydrous organic solvents (e.g., DMF, DMSO); prepare immediately before use (susceptible to hydrolysis).

NHS solution: Dissolve in water or DMF/DMSO.

Reaction buffer: MES buffer (pH 4.5–7.0) is recommended (avoid Tris or amine-containing buffers to prevent reaction interference).

Carboxyl-containing molecules (e.g., proteins, peptides, or small molecules).

Amino-containing molecules (e.g., antibodies, peptides, or amine-modified fluorescent labels).

(2) Carboxyl activation (EDC/NHS step)

Dissolve substrate: Dissolve the carboxyl-containing molecule in a buffer (e.g., 0.1 M MES, pH 6.0); a concentration of 0.1–10 mg/mL is recommended.

Add NHS: Add NHS at 1.2–2 molar equivalents relative to carboxyl groups (e.g., 10 mM final concentration).

Add EDC: Add EDC at 1–5 equivalents relative to carboxyl groups (e.g., 5–20 mM final concentration); react on ice or at room temperature for 15–30 minutes.

Note: EDC is unstable; handle quickly and avoid prolonged exposure to humid environments.

(3) Coupling Reaction (Conjugation with Amino Groups)

Addition of amine-containing molecules: Add the aminated molecule (e.g., antibody) directly to the activated mixture; the amine groups should be in excess (typically 2–10 times the amount of activated carboxyl groups).

Reaction conditions: Incubate at room temperature or 4 °C for 2–4 hours (or overnight; 4 °C is more stable).

pH adjustment: If necessary, adjust the pH to 7.0–8.5 to promote amide bond formation (but avoid pH >8.5 to prevent hydrolysis).

(4) Quenching and Purification

Quenching: Add a quenching agent (e.g., β -mercaptoethanol or glycine) to quench unreacted EDC/NHS.

Purification:

Dialysis: Remove small molecule byproducts by dialysis against PBS or the target buffer.

Size exclusion chromatography (e.g., PD-10 column): Separate the conjugate from unreacted molecules.

Ultrafiltration: Suitable for protein conjugates.

3.2.2.2 Thiol (-SH) Modification: Reacts with maleimide or iodoacetamide for the specific labeling of thiol groups in proteins.

Maleimide: Undergoes a Michael addition reaction with thiol groups to form a stable thioether bond. With a fast reaction rate and high selectivity, it is the most commonly used method for thiol labeling.

Iodoacetamide: Undergoes an alkylation reaction with thiol groups to form a stable thioether bond. It also reacts with other nucleophilic groups (e.g., amino groups or the imidazole group of histidine), but exhibits good selectivity for thiol groups under controlled conditions.

(1) Key pre-experimental preparations

Buffer selection: Use thiol-free buffers; they must not contain reducing agents such as β -mercaptoethanol (BME) or dithiothreitol (DTT), otherwise, the labeling reagent will be consumed.

pH is critical: The optimal pH range is 7.0–7.5. At pH < 6.5, the reaction rate is slow; at pH > 8.0, maleimide may react with primary amines, reducing selectivity.

Avoid amine-containing buffers: Primary amines such as Tris and glycine will compete for the reagent; phosphate-buffered saline (PBS) or HEPES buffer should be used.

Remove metal ions: EDTA (1–5 mM) can be used to sequester metal ions to prevent sulfhydryl oxidation.

Protein pretreatment (reduction to expose sulfhydryl groups): If the target sulfhydryl groups have formed disulfide bonds, they must be reduced first. Add an excess of a reducing agent (e.g., DTT, 5–10 mM) to the buffer. Incubate at 37 °C for 30 minutes. Critical step: Remove excess reducing agent! Use a desalting column (e.g., PD-10), dialysis, or size exclusion chromatography to transfer the protein into a reducing agent-free reaction buffer. This is critical for the success of the experiment.

(2) Reagent preparation:

Freshly prepare a high-concentration stock solution (e.g., 100 mM) by dissolving maleimide or iodoacetamide powder in anhydrous DMSO (dimethyl sulfoxide). Avoid using water, as these reagents are unstable in aqueous solutions. Calculate the molar excess to ensure the labeling reagent is in significant excess relative to the sulfhydryl groups in the protein.

Detailed Procedure

Protocol A: Labeling with Maleimide

- a. Reaction setup: Place the reduced and buffer-exchanged protein sample into a suitable centrifuge tube. The recommended protein concentration is between 1–5 mg/mL.
- b. Add labeling reagent: Based on calculations, slowly add the maleimide-DMSO stock solution to the protein solution. Molar ratio: Typically, an excess of 10:1 to 20:1 (labeling reagent: total sulfhydryl groups) is used. For complex cases, this can be as high as 50:1. Vortex or gently pipette to mix, avoiding the formation of bubbles.
- c. Incubation: Incubate at room temperature (25 °C) or 4 °C protected from light (as some labels and maleimide itself may be photosensitive). Reaction time: Typically 1–2 hours. Excessive reaction time may increase non-specific labeling.
- d. Quenching and purification: Add an excess of a thiol-containing quenching agent to terminate the reaction. The most commonly used are β -mercaptoethanol (BME) or cysteine. The amount of quenching agent added should be significantly higher than that of the remaining labeling reagent (e.g., a final concentration of 10–20 mM). Incubate at room temperature for 5–10 minutes to quench all unreacted maleimide. Use a desalting column or dialysis to remove excess labeling reagent, quenching agent, and byproducts, and to exchange the protein into the desired storage buffer.

Protocol B: Labeling with iodoacetamide

- a. Add the labeling reagent: Add the iodoacetamide-DMSO stock solution. Molar ratio: Typically, an excess of 5:1 to 10:1 (labeling reagent : total sulfhydryl groups) is used. To avoid alkylating other groups, avoid using a large excess.
- b. Reaction incubation: Incubate at room temperature or 4 °C, protected from light. Reaction time: Typically 30 minutes to 1 hour, protected from light.
- c. Reaction termination and purification: After the reaction is complete, purification (dialysis or desalting) can be performed directly to terminate the reaction, as iodoacetamide hydrolyzes slowly. An excess of β -mercaptoethanol or DTT (final concentration of 5–10 mM) can also be added for quenching.

3.2.2.3 Click Chemistry: Introduces alkyne or azide groups for efficient coupling via CuAAC (copper-catalyzed) or SPAAC (strain-promoted).

CuAAC (Copper-Catalyzed Azide-Alkyne Cycloaddition)

Reactants: Azide (N_3) + terminal alkyne ($HC\equiv C-$)

Characteristics: Requires a copper catalyst ($Cu(I)$) and proceeds at a very fast reaction rate, but the copper catalyst may be toxic to biomolecules or cause protein denaturation.

SPAAC (Strain-Promoted Azide-Alkyne Cycloaddition)

Reactants: Azide (N_3) + cyclooctyne (e.g., DBCO, BCN)

Characteristics: Does not require a metal catalyst; it utilizes the ring strain of cyclooctyne to drive the reaction. The reaction rate is slower than CuAAC, but it exhibits excellent biocompatibility, making it highly suitable for live-cell labeling and labile proteins.

(1) Introduction of an azide group ($-N_3$)

Common Methods: Use NHS-ester reagents to react with lysine amino groups on the protein surface.

Reagent example:

NHS-PEG₄-Azide (commonly used; the PEG spacer improves flexibility and water solubility)

Reaction conditions:

Buffer: PBS or HEPES (pH 7.2–8.5); avoid buffers containing amino groups (e.g., Tris, glycine).

Temperature: Room temperature or 4 °C

Time: 1–2 hours

Molar ratio: Reagent:Protein = 3:1 to 20:1 (optimization required to avoid over-labeling, which may lead to protein precipitation or inactivation).

(2) Introduction of an alkyne group ($-C\equiv CH$)

Similarly, using NHS ester reagents:

Reagent example:

NHS-PEG₄-Alkyne (most common)

DBCO-NHS (can be used directly for subsequent SPAAC, skipping the copper-catalyzed step)

Reaction conditions: The conditions are identical to those used for introducing the azide group.

(3) Incorporation of unnatural sugars (used for specific labeling of glycoproteins)

This is a more specific method for labeling glycoproteins on the cell surface.

Method: Add Ac₄ManNAz (to generate azido-sialic acid) or Ac₄GalNAz (to generate azido-galactose) to the cell culture medium.

Mechanism: Cells metabolize these azide-modified sugar analogs and incorporate them into cell surface glycoproteins, thereby specifically introducing azide groups onto the cell surface.

Purification: Following step one, unreacted modifiers and by-products must be removed using a desalting column (e.g., PD-10), dialysis, or ultrafiltration, followed by buffer exchange into the click reaction buffer.

Protocol A: CuAAC (Copper-Catalyzed) Reaction Procedure

Advantages: Extremely fast reaction rate (minutes).

Disadvantages: Risk of copper toxicity and oxidative damage.

a. Prepare reaction buffer: Use degassed PBS or TBS. A small amount of water-soluble ligand may be added to reduce copper toxicity and accelerate the reaction, for example:

THPTA (commonly used and highly efficient)

BTAA (more efficient, compatible with biotin labeling)

TBTA (classic, but poor water solubility)

b. Prepare the reaction stock solution (add in the following order):

Azide- or alkyne-modified protein (final concentration ~10–100 μM)

Molecule with complementary group (e.g., Alkyne-Dye or Azide-Dye) (1.2–2 equiv.)

Ligand (THPTA, BTAA, etc.) (5–10 equiv. relative to Cu)

Finally, add: CuSO_4 (1–5 equiv.) and freshly prepared sodium ascorbate (5–10 equiv. relative to Cu); sodium ascorbate reduces Cu(II) in situ to catalytically active Cu(I).

c. Incubation:

Vortex or invert to mix in the dark at room temperature.

Reaction time: 30 minutes to 2 hours (the reaction is very rapid).

d. Termination and purification:

Add EDTA (final concentration ~5 mM) to chelate copper ions and stop the reaction.

Purify via desalting column, dialysis, or SDS-PAGE to thoroughly remove copper ions, ligands, and unreacted labeling molecules.

Protocol B: SPAAC (Strain-Promoted) Reaction Steps

Advantages: Excellent biocompatibility; requires no metal catalyst and can be performed directly in cell culture media or in vivo.

Disadvantages: The reaction rate is relatively slow (on the scale of hours), and cyclooctyne reagents are expensive.

a. Prepare the reaction system:

Mix the azide-modified proteins (or cells) with DBCO- (or BCN-) labeled molecules (e.g., DBCO-Dye).

Buffer: PBS, HEPES, or complete cell culture medium can be used.

Molar ratio: DBCO reagent : Azide = 1.5:1 to 5:1 (requires optimization).

b. Incubation:

Incubate in the dark at 37 °C (for cells) or at room temperature (for purified proteins).

Reaction time: 1–4 hours (purified proteins); 30 minutes–2 hours (cell surface labeling).

c. Purification:

After the reaction is complete, purify the protein directly using a desalting column, dialysis, or centrifugation.

For cell labeling, wash the cells 2–3 times with PBS to remove unreacted DBCO reagents.

3.2.3 Regulation of Hydrophilicity/Solubility

3.2.3.1 PEGylation (PEG modification): Reduces non-specific adsorption and extends in vivo circulation time.

Polyethylene glycol (PEG) is a hydrophilic polymer with good biocompatibility. Covalently attaching it to drug molecules (mainly proteins, peptides, nucleic acids, and nanoparticles) is like dressing the drug in an "invisibility cloak."

(1) Prolonged circulation time: Through steric hindrance, the PEG chain masks the surface of the drug molecule, making it less susceptible to rapid renal filtration and clearance. It also reduces recognition and phagocytosis by immune cells (such as macrophages), thereby significantly extending its half-life in the bloodstream.

(2) Reduced non-specific adsorption: PEG's high hydrophilicity and dynamic mobility allow it to bind numerous water molecules, forming a "hydration layer" on the drug surface. This water barrier effectively reduces non-specific interactions between proteins, cells, or other biological components and the drug surface, thereby lowering immunogenicity, toxicity, and aggregation.

Step 1: Protein pretreatment

Objective: Ensure the protein is in an optimal state for reaction.

a. Purification and buffer exchange:

Purify the protein to high purity using methods such as HPLC, FPLC, or dialysis.

Key point: Exchange the protein into a reaction buffer that is free of primary amines (e.g., Tris, glycine) and thiols (e.g., β -mercaptoethanol, DTT, GSH). Commonly used buffers include phosphate-buffered saline (PBS, pH 7.2–7.5) or borate buffer (pH 8.0–8.5).

Rationale: Primary amines and thiols react with most PEGylation reagents, consuming the reagents and leading to non-specific modification.

b. Concentration measurement:

Accurately determine the protein concentration using methods such as Bradford, BCA, or UV absorbance to calculate the molar ratio with the PEG reagent.

c. (Optional) Reduction:

If the target modification site is the thiol group (-SH) of a cysteine (Cys), and this thiol has already formed a disulfide bond, it must first be reduced with excess DTT/TCEP, followed by rapid removal of the reducing agent using a desalting column to prevent interference with the subsequent reaction.

Step 2: PEGylation Reaction (Core Step)

Objective: Covalently attach the PEG to specific sites on the protein under controlled conditions.

a. Select the PEG Reagent

PEG molecular weight: Common sizes are 5 kDa, 10 kDa, 20 kDa, and 40 kDa. Higher molecular weight provides better shielding but may more significantly impact protein activity.

PEG type: Linear PEG is most commonly used; branched PEG (Y-shaped PEG) typically provides better shielding and lower immunogenicity.

Reactive functional groups (selected based on the target protein site):

Table 2 Common types of PEG reagents

Target group	PEG reagent type	Reaction conditions	Characteristics
Lysine ϵ -amino group	NHS-PEG (most common)	pH 7.5–8.5, 4 °C to RT	Non-specific modification; may yield a mixture of multiple products.
Cysteine sulfhydryl	Mal-PEG (maleimide PEG)	pH 6.5–7.5, protected from light	High site-specificity, fewer side reactions, good product homogeneity.
N-terminal amine	Aldehyde PEG	pH 5.5–6.5, requires NaBH_3CN reduction	It exhibits some site-specificity, but the reaction conditions are relatively harsh.

b. Reaction procedure

Molar ratio optimization (most critical!) PEG reagent : protein = 3:1 to 20:1 (optimization via pilot experiments required). Low ratios result in poor modification efficiency; excessively high ratios may lead to over-modification, protein inactivation, or precipitation, while complicating purification.

Procedure: a. Place the protein solution in an ice bath. b. Weigh the appropriate amount of PEG reagent and add it slowly to the protein solution in batches while gently stirring or vortexing to prevent protein precipitation due to localized high concentrations. c.

Incubate the reaction mixture protected from light (especially for Mal-PEG) at 4°C or room temperature for 1–4 hours.

Monitoring: Aliquots can be taken at regular intervals to monitor modification progress via SDS-PAGE.

Step 3: Purification and Separation

Objective: Separate the target PEGylated products (mono-PEGylated, di-PEGylated, etc.) and remove unreacted protein, free PEG, byproducts, and salt ions.

a. Ion exchange chromatography (IEX):

Principle: PEGylation alters the protein's surface charge, leading to elution profiles distinct from the unmodified protein.

Effect: Effectively separates products with different degrees of modification (e.g., unmodified, mono-modified, and di-modified).

b. Size exclusion chromatography (SEC/GFC):

Principle: The hydrodynamic volume of PEGylated proteins is significantly larger than that of unmodified proteins, resulting in earlier elution.

Effect: It is the most effective method for removing free PEG and can also separate protein aggregates with different degrees of modification.

c. Ultrafiltration centrifugation:

Principle: Using an ultrafiltration membrane with an appropriate molecular weight cut-off (MWCO) enables protein concentration and removal of small-molecule impurities and salts.

Note: The molecular weight of free PEG may be close to that of the modified protein, making it difficult to remove completely using this method alone; it is often used in combination with other methods.

3.2.3.2 Introduction of amphiphilic molecules: such as phospholipid modification (for cell membrane labeling or nanoparticle encapsulation).

Application: Phospholipid-modified proteins/antibodies (for cell membrane labeling)

Principle: Hydrophobic phospholipids (e.g., DSPE-PEG) are conjugated to hydrophilic macromolecules (e.g., antibodies or proteins) via reactive groups at the PEG terminus (-COOH, -NH₂, -Mal, etc.). The resulting amphiphilic conjugates can spontaneously insert into the lipid bilayer of the cell membrane, thereby achieving cell membrane labeling.

Steps (using EDC/NHS conjugation of DSPE-PEG-COOH with an aminated antibody as an example):

(1) Reagent preparation:

Phospholipid stock solution: Dissolve DSPE-PEG-COOH in chloroform or a chloroform/methanol mixture (e.g., 1–10 mg/mL), aliquot, and store at -20 °C, protected from moisture and light.

Antibody solution: Dissolve the antibody to be labeled (containing -NH₂) in a pH 7.0–7.4 PBS or HEPES buffer at a recommended concentration of 1–5 mg/mL. Ensure the buffer is free of free amino groups (such as Tris, glycine, or sodium azide), as these will interfere with the reaction.

Activating reagent: Freshly prepare aqueous solutions of EDC and NHS (or Sulfo-NHS).

(2) Phospholipid activation (critical step):

Transfer a specific amount of DSPE-PEG-COOH stock solution to a glass test tube, and completely remove the organic solvent using a gentle stream of nitrogen or a vacuum centrifugal concentrator to form a transparent lipid film at the bottom.

Add an appropriate amount of PBS (pH 7.4) or MES buffer (pH 6.0), and vortex vigorously or sonicate (probe sonication in an ice bath is recommended) to form micelles in the aqueous phase.

Add a molar excess (typically 2–5 times the amount of DSPE-PEG-COOH) of EDC and NHS to the micelle solution.

Shake or vortex gently at room temperature for 15–30 minutes to activate the carboxyl groups at the ends of the phospholipids, forming activated NHS esters.

(3) Coupling reaction:

Slowly add the activated phospholipid micelle solution dropwise into the antibody solution. The molar ratio of phospholipid to antibody needs to be optimized (typically starting from 10:1 to 50:1).

Stir the reaction gently for 2–4 hours at 4 °C or room temperature. Avoid vigorous stirring to prevent bubble formation, which can cause protein denaturation.

(4) Purification and characterization:

Objective: To remove unreacted phospholipid micelles, free reagents, and byproducts.

Methods:

Dialysis: Dialyze against PBS (4 °C, 24–48 hours, with multiple buffer changes); suitable for small-scale preparation.

Size Exclusion Chromatography (SEC): Separation using columns such as Superose 6 or Sephacryl S-300 is the most effective method, providing excellent separation of the conjugate (macromolecule) from phospholipid micelles (high aggregation number, early elution) and small-molecule byproducts.

Ultrafiltration: Use ultrafiltration centrifugal filters with a 100 kDa or 300 kDa MWCO, centrifuging multiple times with buffer washes.

Characterization:

SDS-PAGE: Compare molecular weight changes of the antibody before and after modification; upward band shifts or smearing may be observed.

Fluorescent labeling: If using fluorescent phospholipids, conjugation success can be confirmed by monitoring fluorescence in the SEC fractions.

Functional validation: Observe cell membrane labeling efficiency via microscopy following cell incubation experiments.

3.2.4 Targeting Modification

3.2.4.1 Small Molecule Ligands: e.g., folate modification for targeting tumor cells, and biotin for the avidin system.

These two types of modifications generally involve linking small molecule ligands, via their derivatives (typically containing carboxyl or amino groups), to functional groups (amino or carboxyl groups) on the surface of proteins, antibodies, nucleic acids, or nanoparticles through chemical conjugation (e.g., EDC/NHS).

Folic Acid: Targets the folate receptors that are highly overexpressed on the surface of many tumor cells. Commonly used in targeted drug delivery and tumor imaging probe development.

Biotin: Exhibits extremely high affinity ($K_d \sim 10^{-15}$ M) for avidin or streptavidin. Used to construct robust detection, purification, and capture systems (e.g., ELISA, fluorescence detection, protein purification).

Since the conjugation chemistry of folic acid and biotin is very similar, the following general protocol uses the common "conjugation of carboxyl-modified ligands to amine-containing proteins" as an example, while highlighting their specific differences.

General Experimental Protocol (Example: Antibody modification with folate-NHS or biotin-NHS esters)

(1) Reagent and Material Preparation

a. Amine-containing macromolecules: Proteins, antibodies, or nanoparticles to be labeled (dissolved in PBS, pH 7.4, concentration ≥ 1 mg/mL). Ensure the buffer is free of primary amines (e.g., Tris, glycine, sodium azide).

b. Ligand derivatives:

For folic acid: Folate-PEG-NHS or Folic Acid-NHS Ester. The PEG chain can increase flexibility and reduce steric hindrance.

For biotin: Biotin-PEG-NHS or Biotin-NHS ester (e.g., Sulfo-NHS-LC-biotin, which has better water solubility).

c. Reaction buffer: Anhydrous DMSO (for dissolving the ligand-NHS ester), PBS (pH 7.4), or borate buffer (pH 8.5). A slightly alkaline pH (7.4–8.5) facilitates the reaction between the NHS ester and amine groups.

d. Purification equipment: Dialysis tubing (MWCO 10–14 kDa), desalting column (e.g., PD-10), or centrifugal ultrafiltration unit (MWCO 50–100 kDa).

(2) Procedure

a. Ligand solution preparation:

Prepare a high-concentration stock solution (e.g., 10–50 mM) of Folate-NHS or Biotin-NHS ester powder in anhydrous DMSO. Prepare immediately before use to avoid hydrolysis.

b. Reaction molar ratio determination:

Recommended starting point:

Biotinylation: Typically use a Biotin-NHS : protein ratio of 5:1 to 20:1 (mol/mol). For detection applications, a labeling degree of 3–5 biotins per protein molecule is a common target.

Folate modification: The ratio may be higher, typically 10:1 to 40:1 (mol/mol), to ensure sufficient targeting ligand density.

Calculate the required volume of the ligand-NHS ester.

c. Coupling reaction initiation:

Place the protein solution on ice.

Slowly add the calculated volume of ligand-NHS ester/DMSO solution dropwise to the protein solution while continuously and gently vortexing or stirring to ensure uniform mixing and prevent protein precipitation due to localized high DMSO concentrations.

The final concentration of DMSO in the reaction system should be $\leq 10\%$.

Incubate the reaction mixture in the dark at room temperature (22–25 °C) for 2 hours, or at 4 °C overnight (milder conditions to reduce protein aggregation).

d. Reaction quenching:

Add an excess of glycine or Tris buffer (pH 8.0) to a final concentration of 50–100 mM, and incubate at room temperature for 10 minutes. The free amino groups of glycine quench unreacted NHS esters.

e. Purification:

Purpose: Remove small molecules such as unreacted ligands, DMSO, and quenchers.

Methods:

Dialysis: PBS (4 °C, 24–48 hours, change buffer 3–4 times). Economical but time-consuming.

Desalting column/size exclusion chromatography (preferred): Use a PD-10 column or similar product, following the manufacturer's instructions. Rapid (15–20 minutes) and efficient; this is the most commonly used method.

Ultrafiltration/Centrifugation: Use an ultrafiltration tube with an appropriate molecular weight cut-off (MWCO), centrifuge several times, and wash with PBS.

f. Product characterization and storage:

Concentration determination: Use the BCA or Bradford method to determine the concentration of the purified product.

Degree of modification analysis:

Degree of biotinylation: Quantitatively determine using a HABA/Avidin kit, or visualize via SDS-PAGE followed by Western blot with streptavidin-HRP incubation.

Degree of folate modification: Can be analyzed via UV-Vis spectroscopy. Folic acid has characteristic absorption peaks at approximately 280 nm and 360 nm; the degree of labeling can be estimated by comparing the absorbance with that of the original protein.

Storage: Store in aliquots at 4 °C or -20 °C, protected from light.

3.2.5 Stimuli-Responsive Modification

Enzyme substrate linkage: A fluorophore and a quencher are connected via an enzyme-specific sequence to achieve activatable imaging (e.g., caspase-3 probes). The core of such probes involves the covalent attachment of a fluorophore and a quencher through an enzyme-specific substrate peptide.

The following are the detailed steps, principles, and key considerations for designing and synthesizing such probes, using Caspase-3 as an example.

3.2.5.1 Core Design Principles

"Off-State": When the fluorophore and quencher are in close spatial proximity, fluorescence resonance energy transfer (FRET) or static quenching occurs. Energy is transferred from the fluorophore to the quencher and dissipated as heat or other forms, resulting in no detectable fluorescence.

"On-State": When the peptide chain connecting the two is cleaved by a specific enzyme (e.g., Caspase-3), the fluorophore and the quencher are physically separated. The FRET process is disrupted, restoring the fluorophore's fluorescence and generating a detectable signal.

Selection of Key Components:

(1) Fluorophore: e.g., FAM, Cy3, Cy5, TAMRA. Excitation/emission wavelengths must be compatible with the imaging system.

(2) Quencher: Select a quencher with maximum spectral overlap with the emission spectrum of the fluorophore to achieve efficient quenching.

Common quenchers for FAM: BHQ-1, Dabcyl.

Common quencher for Cy3: BHQ-2.

Common quenchers for Cy5: BHQ-3, QSY21.

(3) Enzyme-Specific Sequence:

Caspase-3: DEVD (amino acid sequence: Asp-Glu-Val-Asp)

MMP-2/9: GPLGVRG

Thrombin: LVPRGS

Chemical modification is typically performed at the C-terminus of the sequence for attachment to the quencher, while the N-terminus is conjugated to the fluorophore.

3.2.5.2 Experimental Procedures (Example: Solid-Phase Synthesis)

The synthesis of such probes is typically performed using Fmoc solid-phase peptide synthesis (SPPS) on an automated peptide synthesizer. The general steps are as follows:

(1) Resin pretreatment and coupling of the first amino acid

Load the Wang resin or Rink Amide resin pre-loaded with the first amino acid (typically the C-terminal residue near the quencher, such as Asp(D)) into the synthesis column, and swell with DMF.

Deprotect the Fmoc group using a piperidine/DMF solution to expose the free amino group.

(2) Stepwise elongation of the peptide chain

Cycle steps: a. Coupling: Mix the next Fmoc-protected amino acid (e.g., Val(V)), an activator (e.g., HBTU/HOBt), and a base (e.g., DIPEA) in DMF, then introduce the mixture into the reaction column to react with the free amino groups on the resin. b. Washing: Wash the resin with DMF to remove excess reagents. c. Deprotection: Remove the Fmoc protecting group from the newly coupled amino acid using a piperidine/DMF solution to expose the amino group for the next cycle.

Repeat this cycle to synthesize the peptide chain sequentially in the order of Quencher-D-E-V-D-Fluorophore. The sequence is crucial.

(3) Conjugation of the fluorophore

The fluorophore is attached to the N-terminus of the peptide chain (following Fmoc deprotection of the final amino acid) by reacting it with an NHS ester-modified fluorophore (e.g., FAM-NHS) under basic conditions.

(4) Attachment of the quencher

If the quencher (e.g., BHQ-COOH) is a carboxylic acid, it is coupled to the first amino acid on the resin using a method similar to step 3 (EDC/HOBt) after deprotection.

(5) Cleavage and deprotection

Upon completion of synthesis, the full-length probe is cleaved from the resin using a cleavage cocktail (e.g., TFA:TIS:H₂O = 95:2.5:2.5), which simultaneously removes all side-chain protecting groups.

The crude product is then precipitated with cold diethyl ether and collected by centrifugation.

(6) Purification and characterization

Purification: Purify using preparative high-performance liquid chromatography (HPLC), typically employing a C18 reverse-phase column with acetonitrile/water (containing 0.1% TFA) gradient elution.

Characterization Use analytical HPLC and mass spectrometry (MS) to verify the purity and molecular weight of the purified product.

(7) Probe Validation (Mandatory)

Spectroscopic Characterization

Dissolve an appropriate amount of the purified probe in PBS and measure its fluorescence spectrum. A very low fluorescence signal should be observed, demonstrating high quenching efficiency.

Add active recombinant Caspase-3 enzyme to the solution and incubate at 37 °C for 1–2 hours.

Measure the fluorescence spectrum again. Significant fluorescence enhancement (tens to hundreds of fold) should be observed.

Specificity Validation Incubate the probe with inactivated enzyme or other unrelated proteases; no fluorescence activation should occur.

3.2.5.3 Alternative: Purchase commercial probes and perform in-house validation

For most biological laboratories, synthesizing probes in-house is impractical. A more common workflow is:

(1) Purchase commercial probes: Purchase probes such as FAM-DEVD-BHQ1 or Cy5-DEVD-QSY21 from reputable suppliers (e.g., AAT Bioquest, Thermo Fisher, MedChemExpress).

(2) Performance validation:

Quenching efficiency: Measure the baseline fluorescence intensity ($F_{\min}$) of the probe itself.

Full activation control: Incubate with excess enzyme until fluorescence plateaus, and measure the maximum fluorescence intensity ($F_{\max}$).

Quenching efficiency (%) = $[1 - (F_{\min} / F_{\max})] * 100\%$. High-quality probes should exhibit a quenching efficiency >95%.

(3) In vitro and in vivo experiments:

Cell imaging: Introduce the probe into cells (e.g., via microinjection, transfection reagent, or electroporation) and observe the fluorescence signal triggered by caspase-3 activation during apoptosis using confocal microscopy.

In vivo imaging: Near-infrared probes (e.g., Cy5-DEVD-BHQ3) can be administered to tumor-bearing mice via intravenous injection, and the apoptosis activation signal at the tumor site can be observed using an in vivo fluorescence imaging system.

3.2.5.4 Key Considerations and Optimization

Linker: Incorporating an appropriate linker (e.g., aminocaproic acid, Ahx) between the fluorophore/quencher and the peptide chain reduces steric hindrance and improves cleavage efficiency.

Solubility: The entire molecule may be hydrophobic; if necessary, introduce charged amino acids (e.g., Lys, Arg, Glu) into the sequence to improve water solubility.

Enzymatic kinetics: Optimize incubation time, temperature, and enzyme concentration to ensure effective cleavage of the probe.

Cytotoxicity: Ensure that the probe itself does not exhibit significant cytotoxicity so as not to interfere with normal biological processes.

Control experiments: Always include a negative control without the enzyme and a control group treated with a broad-spectrum caspase inhibitor (e.g., Z-VAD-FMK) to demonstrate the specificity of the signal.

3.2.6 Metal Ion Chelation

The introduction of chelating groups (such as DOTA, NOTA, or EDTA) for metal ion sensing or multimodal imaging (e.g., MRI/fluorescence) is a common design strategy for molecular probes. Such modifications are generally intended to achieve one of two objectives:

Metal ion sensing: Upon binding to specific metal ions (such as Zn^{2+} , Cu^{2+} , or Ca^{2+}), the chelating group causes a change (enhancement or quenching) in the signal of the attached fluorophore.

Diagnostic imaging: Chelating groups are used to chelate radioactive metals (such as ^{68}Ga or ^{64}Cu for PET) or paramagnetic metals (such as Gd^{3+} for MRI), while the attached fluorophore is used for optical imaging, enabling dual-modality (e.g., PET/fluorescence or MRI/fluorescence) imaging.

Using the classic DOTA as an example, the following section details the specific steps for its modification and application.

3.2.6.1 Core Design Principle

DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid): A macrocyclic polydentate ligand with extremely high affinity and stability for trivalent metal ions (such as Gd^{3+} , Ga^{3+} , Lu^{3+} , In^{3+}).

Modification strategies:

DOTA contains four carboxyl groups, one of which can be chemically modified to generate reactive derivatives such as DOTA-NHS ester or DOTA-maleimide, which then react with amino or thiol groups on biomolecules.

The structure of the final constructed probe is typically: Targeting molecule/fluorophore - Linker - DOTA.

Workflow: Chemical conjugation, followed by metal chelation, and finally purification. The chelation step is usually performed at high temperatures to ensure high efficiency.

3.2.6.2 Experimental procedure (Example: DOTA-NHS ester modification of an aminated antibody for subsequent Gd³⁺ chelation and MRI imaging)

Phase I: Antibody modification with DOTA (chemical conjugation)

(1) Reagent preparation:

DOTA derivative: DOTA-NHS ester (commercially available, e.g., from Macrocyclics or Bolumina). Important Note: Dissolve in anhydrous DMSO to prepare a 10–50 mM stock solution immediately before use.

Molecule to be modified: Purified antibody (IgG), dissolved in an amine-free buffer (e.g., 0.1 M HEPES, pH 8.5; or 0.1 M MES, pH 6.0). Recommended concentration >1 mg/mL. Avoid using amine-containing buffers such as Tris or glycine.

Purification equipment: PD-10 desalting column (GE Healthcare) or centrifugal filter unit (MWCO 50 kDa).

(2) Conjugation reaction:

Place the antibody solution on ice.

Calculate the amount of DOTA-NHS ester required. The molar ratio (DOTA:Ab) needs to be optimized, typically starting from 10:1 to 50:1, to avoid antibody aggregation or inactivation caused by over-modification.

Add the DOTA-NHS ester/DMSO solution slowly, dropwise, to the antibody solution while maintaining gentle, continuous vortexing.

Incubate the reaction mixture in the dark at room temperature (~25 °C) for 2 hours, or overnight at 4 °C (a milder condition).

(3) Purification:

Using a PD-10 desalting column with PBS (pH 7.4) as the eluent, follow the manufacturer's instructions to collect the protein peak.

This step is intended to remove unreacted DOTA-NHS ester, hydrolysis products, and DMSO.

Collect the purified DOTA-Ab conjugate, determine the concentration (BCA assay), and store in aliquots at 4 °C or -20 °C.

Phase II: Metal ion chelation (using Gd^{3+} as an example)

(1) Reagent preparation:

Metal salt: $GdCl_3$ solution (dissolved in a weakly acidic aqueous solution, such as 50 mM HCl, to prevent hydrolysis and precipitation).

Buffer: 0.1–0.5 M ammonium acetate buffer (pH 5.5–6.0). This pH range is optimal for the chelation of Gd^{3+} with DOTA.

Competing chelator (optional): Citric acid, used to scavenge free Gd^{3+} that has not been successfully chelated.

(2) Chelation reaction:

Dilute the purified DOTA-Ab conjugate or perform buffer exchange (via centrifugal ultrafiltration) using ammonium acetate buffer (pH 5.5).

Add a 10–20 fold molar excess of $GdCl_3$ solution.

Incubate at 37–50 °C for 1–2 hours (higher temperatures help overcome the chelation energy barrier and improve yield). Vortex occasionally.

(3) Purification and characterization:

Purpose: To remove free, unchelated Gd^{3+} , which is critical for in vivo applications due to the toxicity of free Gd^{3+} .

Methods:

Dialysis: Dialysis against PBS (containing 1–5 mM EDTA, followed by thorough dialysis against PBS without EDTA). EDTA acts as a strong chelating agent to remove free Gd^{3+} .

PD-10 desalting column: Use PBS as the mobile phase; it is rapid and effective.

Ultrafiltration centrifugation: Use a 50 kDa MWCO ultrafiltration tube and perform multiple wash-centrifugation cycles with PBS.

Verification of chelation efficiency:

Inductively coupled plasma mass spectrometry (ICP-MS): The gold standard method. By separately measuring the Gd and protein content (via S or N element analysis) in the purified conjugate, the number of Gd^{3+} ions chelated per antibody molecule can be precisely calculated.

Radiolabeling simulation: Perform chelation using radioisotopes (e.g., ^{153}Gd , ^{111}In), followed by size exclusion chromatography (SEC) coupled with a radioactivity detector to quantify chelation efficiency and stability via peak analysis.

Phase III: Functional Validation (Example: MRI/Fluorescence Dual-Modality Probe)

(1) Measurement of MRI relaxivity:

Place the Gd-DOTA-Ab conjugate at various concentrations into an MRI scanner or an NMR relaxometer.

Measure its T_1 relaxation time.

Plot $1/T_1$ (s^{-1}) against Gd concentration (mM); the slope represents the relaxivity (r_1), a key metric for assessing MRI contrast enhancement efficiency.

(2) Fluorescence characterization:

If the antibody is fluorescently labeled or co-modified with DOTA and a fluorophore, measure the fluorescence spectra before and after chelation to ensure properties are unaffected by the process.

(3) In vitro/in vivo imaging:

In vitro targeting verification: Incubate with cells expressing the target antigen and observe via confocal microscopy whether fluorescence is enriched on the cell membrane (demonstrating targeting ability and fluorescence function).

In vivo MRI imaging: Inject the probe into animal models and scan using an MRI device to observe whether the T_1 -weighted signal in the tumor or other target tissues is enhanced.

Ex vivo fluorescence imaging: After euthanasia, organs are harvested and fluorescence distribution is observed using an IVIS to correlate with MRI results.

3.3 Imaging Data Analysis and Quantification Methods for Fluorescent Probes

From each ROI, the software typically provides the following key data:

(1) Total efficiency or Total flux [photons/s]: The sum of signals from all pixels within the ROI. This is the most commonly used and reliable metric, as it reflects the total amount of signal and is less sensitive to ROI size.

- (2) Average radiance [p/s/cm²/sr]: The average signal value of all pixels within the ROI. This indicates the "concentration" or "brightness" of the signal. This value may be biased when ROI sizes are inconsistent.
- (3) Maximum radiance: The value of the brightest pixel within the ROI. Used to evaluate peak signal intensity.
- (4) ROI area [cm² or pixels]: Records the size of the ROI for auxiliary assessment.

3.3.1 Pharmacokinetic Analysis

If long-term, multi-timepoint dynamic imaging is performed, time-signal intensity curves can be plotted and pharmacokinetic-like parameters calculated:

Time to Peak (T_{max}): The time at which the signal reaches its peak.

Peak Intensity (I_{max}): The peak value of the signal (usually expressed as TBR).

Area Under the Curve (AUC): The area under the signal intensity-time curve from time zero to a specific time point, reflecting the total exposure of the probe in the target area.

Note: Target-to-Background Ratio (TBR) is the most critical and meaningful quantitative metric in in vivo fluorescence imaging. It eliminates the effects of animal autofluorescence, non-specific accumulation of the probe, and instrumental noise.

Formula: $TBR = (\text{Mean Radiant Efficiency of Target ROI}) / (\text{Mean Radiant Efficiency of Background ROI})$

A TBR >2 is generally considered to indicate a significant specific signal. A higher TBR indicates better probe specificity and a higher signal-to-noise ratio.

When performing comparisons between groups, TBR is more convincing than absolute fluorescence values.

3.3.2 Ex Vivo Verification and Correlation Analysis

The ultimate verification for in vivo imaging is to correlate the imaging data with the "gold standard" ex vivo data.

After imaging is completed, the animals are euthanized immediately, and the target tissues and major organs (heart, liver, spleen, lung, kidney) are harvested.

A small animal imaging system is used to image the ex vivo organs and calculate the total radiant efficiency for each organ.

Furthermore, tissues can be homogenized, and precise absolute fluorescence values can be measured using a fluorescence spectrophotometer.

Finally, perform a correlation analysis (such as calculating the Pearson correlation coefficient) between the ex vivo measured fluorescence values and the TBR or total flux measured by in vivo imaging; a strong positive correlation can significantly enhance the credibility of the in vivo data.

3.4 Typical Case Studies

Ex vivo distribution of Mn_3O_4 nanozymes-Cy3

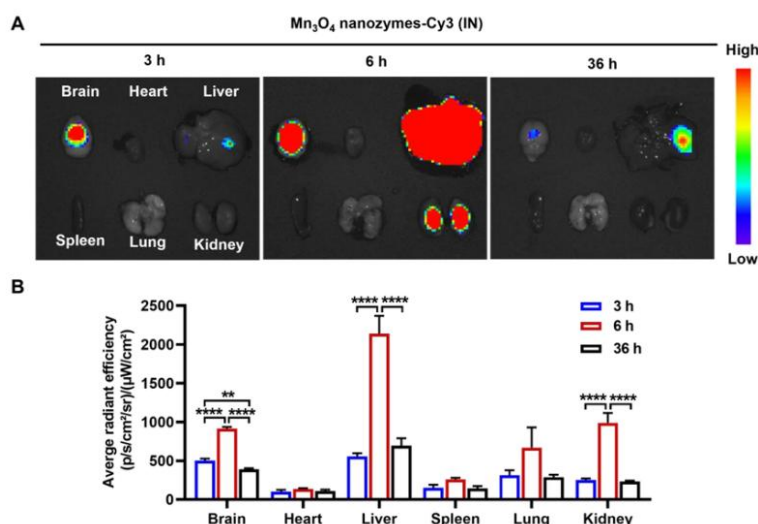


Figure 8 Ex Vivo Distribution of Nanomedicine

Through long-term in vivo imaging at multiple time points, this study systematically tracked and analyzed the spatiotemporal distribution dynamics and metabolic patterns of Mn_3O_4 nanozymes-Cy3 probes in mouse models. Specifically, at several preset time points (3 h, 6 h, and 36 h) following intravenous injection, 2D distribution images of the Cy3 fluorescence signal across major organs were acquired using a small animal in vivo fluorescence imaging system.

By performing semi-quantitative analysis on the images, the researchers plotted the fluorescence intensity-time curves of the probes in various organs (including the liver, kidneys, spleen, lungs, heart, and brain). The results showed that significant signal enrichment was observed in the brain as early as 3 hours after probe injection, demonstrating that Mn_3O_4 nanozymes-Cy3 can accumulate in the brain; subsequently, marked fluorescence enhancement was also visible in the liver and kidneys, suggesting that the probes may be excreted from the body via hepatorenal clearance. In contrast,

the signal intensity in organs such as the heart, lungs, and spleen remained consistently low, indicating minimal non-specific retention of the probe in these tissues. Notably, as time progressed to 36 hours, the fluorescence signal in the liver and kidney regions showed a slow downward trend, indicating that the probe can be gradually cleared by the body over time, demonstrating good bioclearability, which is a positive indicator for the safety of its future biomedical applications. Furthermore, fluorescence imaging and quantitative analysis of isolated major organs further verified and supplemented the in vivo imaging results, precisely revealing the actual accumulation of the probe in different tissues and providing critical data support for a comprehensive evaluation of the in vivo pharmacokinetics and biodistribution characteristics of this nanozyme probe.^[3]

Chapter 4 Application of In Vivo Imaging in Exosome Tracking and Targeting

4.1 Common Models for Exosome Tracing

Exosomes (or small extracellular vesicles, sEVs; diameter approx. 30–150 nm) are lipid bilayer vesicles actively secreted by cells, serving as natural messengers for intercellular communication and are enriched with proteins, nucleic acids (miRNA, mRNA), and lipids. Acting like microscopic "letters," they precisely convey information throughout the body, playing critical roles in tumor development and progression, immune regulation, tissue regeneration, and neurodegenerative diseases.

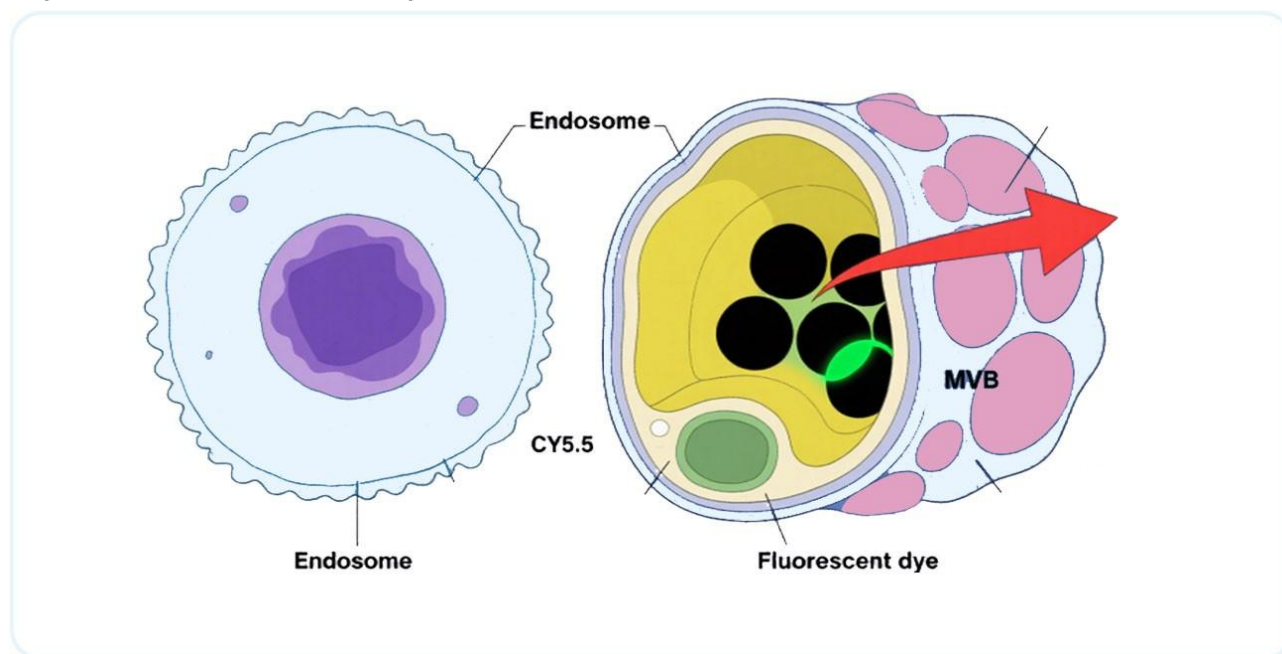


Figure 9 Schematic of exosome tracing models

Conventional research methods, such as transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and biochemical assays, provide static morphological and molecular information but fail to address a core question: once these "messengers" enter a living organism, where do they distribute, how are they cleared, and can they selectively home to specific lesions?

The application of small animal in vivo imaging has provided a breakthrough solution to this challenge. By labeling exosomes with fluorescent or bioluminescent probes, their in

vivo behavior can be converted into visualizable and quantifiable optical signals, allowing researchers to track the entire process of exosomes—from injection to distribution, homing, and clearance—in real-time at the organismal level. This provides a powerful tool for gaining deep insights into the biological functions of exosomes, evaluating their delivery efficiency as drug carriers, and advancing the translational application of exosomes in diagnostics and therapeutics.

4.2 Construction and Labeling Strategies of Exosome Tracing Systems

The key to achieving in vivo imaging of exosomes lies in constructing a stable, specific, and high-brightness tracing system. This primarily relies on two core strategies: **genetic/endogenous labeling** and **exogenous labeling**. These two methods each have their advantages and disadvantages and must be combined with strict experimental controls to ensure data accuracy.

4.2.1 Genetic/Endogenous Labeling: Achieving Long-Term, Stable Endogenous Tracing

Principle: This method employs genetic engineering to stably introduce a reporter gene into the genome of donor cells, where the encoded reporter protein (such as a fluorescent protein or luciferase) is expressed as a fusion with an exosomal membrane protein (such as CD63 or CD9). When cells secrete exosomes, these vesicles naturally carry fluorescent or bioluminescent tags. This strategy endows exosomes with imaging capabilities at the biological source and is considered the most reliable and specific tracing method.

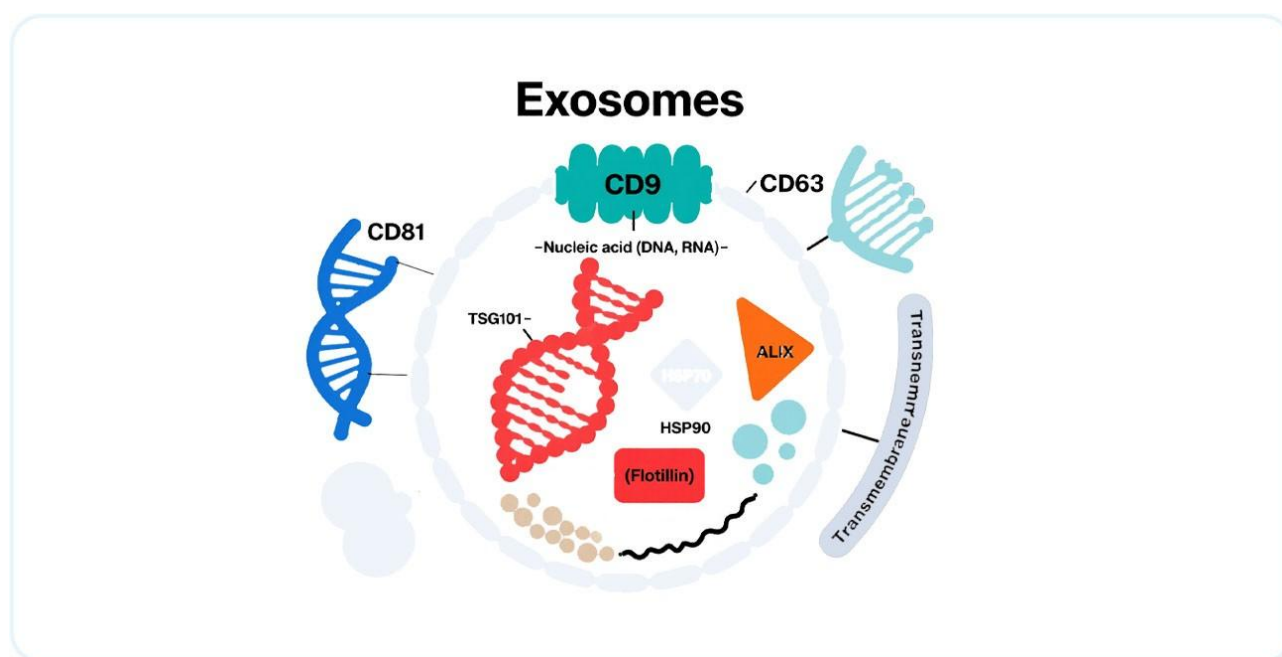


Figure 10 Schematic of In Vivo Exosome Imaging and Tracing Model

Advantages and Applications:

High specificity and stability: Because the labeling molecule is an integral part of the exosomal membrane, it does not dissociate or transfer to other cells, fundamentally eliminating the risk of false positives associated with exogenous labeling. This is crucial for studying the slow accumulation or long-term effects of exosomes in vivo, such as their role in the pre-metastatic niche.

Low background signal: The bioluminescence (Luciferase) system does not emit light in the absence of a substrate, and the autoluminescence background of living tissues is extremely low, giving this labeling strategy an exceptionally high signal-to-noise ratio and sensitivity. This enables researchers to effectively detect trace, low-abundance exosomes in vivo, even in difficult-to-reach deep tissues such as the brain or heart. For instance, a study successfully traced the in vivo distribution of cardiac-derived exosomes by constructing a mouse model with cardiomyocyte-specific expression of NanoLuc-CD63, revealing their role in cardiac diseases.

Common Probes and Practical Considerations:

Fluorescent proteins (e.g., EGFP, mCherry): Suitable for in vitro confocal imaging to verify the cellular internalization and subcellular localization of exosomes. However, due to poor tissue penetration, they are not suitable for deep-tissue in vivo imaging.

Luciferases (e.g., Firefly Luc2, NanoLuc): High brightness and low background make them the preferred choice for in vivo tracking. NanoLuc is particularly notable for its ultra-high brightness and stability, effectively overcoming bioluminescent photon attenuation in deep tissues.

Practical advice: When constructing fusion expression vectors, appropriate linkers should be selected to avoid interfering with protein conformation. Furthermore, prior to in vivo experiments, successful fusion protein expression must be confirmed via **Western blot**, and exosome size and concentration must be verified by **NTA** (nanoparticle tracking analysis) to ensure that labeling has not significantly altered their physical properties.

4.2.2 Exogenous Labeling: Flexible and Rapid, but Requires Rigorous Controls

Underlying principle: This method involves labeling exosomes in vitro, followed by purification and administration back into the animal. This strategy is widely adopted for its simplicity and lack of requirement for genetic modification, making it particularly suitable for studying clinical samples or exosomes from specific sources.

Common methods:

Lipophilic membrane dyes (e.g., DiR, DiI, PKH26): These dyes utilize hydrophobicity to insert into the exosomal membrane. This method is simple to perform, yields a strong signal, and is suitable for rapid screening of the general in vivo distribution of exosomes.

Chemical conjugation (e.g., NHS-ester): Fluorescent dyes (e.g., Cy5.5, IRDye) are linked to lysine residues on the surface of exosomes via covalent bonds. This method offers higher specificity than lipophilic dyes and reduces the risk of dye dissociation.

Click chemistry: Involves pre-labeling exosomes in vitro with azide ($-N_3$) or alkyne ($-C\equiv CH$) groups, followed by conjugation with a fluorescent dye bearing a complementary group via a highly efficient and specific click reaction (e.g., SPAAC). This method requires no genetic modification while maintaining high specificity, providing a new alternative for exosome tracking.

Advantages and core risks:

High flexibility: Can be used for exosomes from any source, without the need for complex genetic modification.

Core risk: Lipophilic dyes carry a significant risk of **false positives**. Dyes may leach from exosomes, form **aggregates**, or bind to **lipoproteins** in the plasma, and be rapidly cleared by organs such as the liver and spleen, leading researchers to mistakenly conclude that exosomes have targeted these organs.

Essential Controls and Validation:

To avoid false positives, the following rigorous controls must be performed:

- i. **"Dye-only Control":** Treat exosome-free buffer with the same dose of dye and subject it to the same purification process. Inject this "dye-only" control into mice; if a signal appears during in vivo imaging, it indicates that the signal may originate from dye aggregates.
- ii. **Density gradient validation:** Subject labeled exosome samples to sucrose or iodixanol density gradient ultracentrifugation to determine if the fluorescence signal coincides with the exosome density peak (1.10–1.21 g/mL).
- iii. **Functional validation:** For chemical conjugation, evaluate exosome **internalization efficiency** and **biological function** before and after labeling to ensure the process has not compromised biological activity.

4.3 Exosome Isolation, Purification, and Administration Design

4.3.1 Isolation and Quality Control: Ensuring Exosome Purity

The isolation and purification of exosomes are the cornerstone of successful downstream tracing experiments. Low sample purity, involving contamination with other extracellular vesicles (e.g., microvesicles) or free proteins, will severely interfere with tracing results.

Isolation Methods:

Differential Ultracentrifugation: Based on differences in particle size and density, exosomes are isolated through multiple cycles of high-speed centrifugation. This method is relatively simple to perform but yields lower purity, with potential contamination from other proteins or vesicles.

Density Gradient Ultracentrifugation: Samples are layered on a gradient medium such as sucrose or iodixanol and centrifuged, allowing exosomes to sediment to their specific buoyant density (1.10–1.21 g/mL). This yields high-purity exosomes and is currently the recognized **gold standard**.

Size Exclusion Chromatography (SEC): Separates exosomes based on their particle size using specialized gel columns, effectively removing free proteins.

Immunoaffinity capture: Captures exosomes using specific antibodies (e.g., CD63, CD9), which achieves high-purity isolation but may introduce bias.

Quality control: To ensure experimental reproducibility, every batch of exosome samples must undergo rigorous quality control. According to the requirements of the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines, the following key data must be recorded:

Particle size and concentration: Use nanoparticle tracking analysis (NTA) or tunable resistive pulse sensing (TRPS) to measure the particle size distribution and particle concentration of exosomes.

Protein content: Determine total protein levels using BCA or other protein quantification methods.

Marker analysis: Verify the expression of specific exosome markers (e.g., CD63, TSG101) via **Western blot**, and detect non-exosomal contamination markers (e.g., the endoplasmic reticulum protein Calnexin).

Electron microscopy: Use transmission electron microscopy (TEM) to confirm the typical cup-shaped or spherical morphology of exosomes.

4.3.2 Route of Administration and Dose Design: Key Factors Affecting In Vivo Fate

The in vivo distribution and clearance rate of exosomes are greatly influenced by the route of administration. Choosing an appropriate route of administration is a prerequisite for achieving precise tracing and targeted delivery.

Route of administration:

Intravenous (IV): This is the most commonly used route of administration, allowing exosomes to rapidly enter the systemic circulation. However, the majority of exosomes are rapidly cleared by the reticuloendothelial system (RES), which is rich in macrophages, particularly in the liver and spleen. Studies have shown that the half-life of exosomes in circulation after intravenous injection is typically only a few minutes to a few hours^[4].

Intraperitoneal (IP) injection: Exosomes are absorbed via the lymphatic and portal vein systems, resulting in slower entry into the systemic circulation; this reduces rapid clearance by the RES and provides local effects on intra-abdominal organs.

Local injection: Such as intratumoral (IT) or intra-articular injection, is suitable for evaluating local therapeutic efficacy. It avoids clearance issues associated with systemic distribution and maximizes the accumulation of exosomes at the target site.

Dose design: To ensure the comparability of results across different studies, it is recommended to report doses in terms of **particle number (particles/dose)** or **total**

protein mass (μg EV protein/dose). Providing both pieces of information in reports, along with the particle-to-protein ratio, can offer readers more comprehensive information.

4.4 Imaging Data Analysis and Quantitative Methods

In vivo imaging provides a wealth of data, but how to accurately process and quantify this data directly determines the reliability of the research conclusions.

4.4.1 Imaging Modalities: Comparison of Strengths and Weaknesses

Bioluminescence Imaging (BLI):

Advantages: Extremely low background signal, high signal-to-noise ratio, and excellent sensitivity, making it suitable for tracking low-abundance exosomes.

Disadvantages: Luminescence signals attenuate with tissue depth and depend on the in vivo distribution and penetration of the substrate (e.g., D-luciferin); thus, it is not an absolute quantification method.

Near-Infrared Fluorescence Imaging (NIRF):

Advantages: Near-infrared light (700–900 nm) has strong penetrating power, is minimally affected by tissue autofluorescence and light scattering, and is suitable for deep organ imaging.

Disadvantages: Fluorescence signals are subject to tissue attenuation and scattering; additionally, ex vivo labeled dyes (e.g., DiR) may pose a risk of false positives.

PET/SPECT:

Advantages: Provides **absolute quantitative data** (%ID/g); signals are not limited by tissue depth, making it the "gold standard" for evaluating biodistribution.

Disadvantages: Equipment is expensive, requires radioisotopes, and has high technical requirements for operation.

4.4.2 Quantitative Metrics and Data Processing

To ensure the standardization and comparability of results, the following metrics are recommended:

Total Flux: Measured in **photons/s**; reflects the total photon output within the ROI (region of interest) and is the most reliable quantitative metric for bioluminescence imaging.

Mean Radiance: Measured in **p/s/cm²/sr**; reflects signal intensity, facilitating comparisons between different experimental groups.

Target-to-Background Ratio (TBR): $TBR = \text{target signal} / \text{background signal}$; it eliminates the effects of instrumental differences and non-specific background signal, making it the most critical metric for measuring probe specificity.

Standardization Procedures and Statistics:

Acquisition of raw images: Use standardized exposure times and excitation/emission filter settings.

ROI definition: Draw ROIs on the images using anatomical landmarks or tumor margins as references.

Background subtraction: Subtract the signal from non-injection sites or dye-blank groups from the ROI signal to obtain the net signal.

Signal normalization: Normalize the data according to injection dose, animal body weight, or total particle number to eliminate individual variability.

Kinetic curves: Plotting ROI signal over time allows for the calculation of the in vivo **half-life (t_{1/2})** and **area under the curve (AUC)** of exosomes, reflecting their pharmacokinetic behavior.

Statistical recommendations: To obtain robust statistical results, a minimum of **5 mice** per group is recommended for biological replicates. Report results using mean $\pm$ standard error of the mean (SEM) or standard deviation (SD), and specify the statistical test method (e.g., t-test or ANOVA).

4.5 Exosome Targeting Mechanisms and Engineering Strategies

The ultimate value of in vivo imaging lies in validating the targeted delivery of exosomes. Understanding their natural homing mechanisms and implementing engineering modifications are key to achieving precision therapy.

4.5.1 Natural Targeting: Integrin-Mediated Organotropism

As previously mentioned, the pioneering study by Hoshino et al.^[5] confirmed the key role of integrins on the surface of tumor-derived exosomes in mediating organotropism. Their work not only explained the biological mechanism of tumor metastasis but also provided molecular targets for the design of "natural" targeted drug vehicles. For example, exosomes with specific integrin profiles can be utilized to naturally deliver drugs to the lungs or liver.

4.5.2 Surface Engineering Strategies: Achieving Enhanced Targeting

To enhance the targeting capabilities of exosomes, researchers can employ surface engineering modifications. In vivo imaging is a core tool for verifying the efficacy of these modification strategies.

Genetic encoding of targeting peptides/antibody display: Through genetic engineering, targeting ligands (such as the **RVG peptide**, which targets neurons; or the **RGD peptide**, which targets tumor vasculature) are fused onto exosomal membrane proteins (such as Lamp2b or CD63). Subsequently, in vivo imaging can be used to track whether these engineered exosomes efficiently accumulate at the intended target (e.g., brain tumors).

Chemical conjugation/PEGylation: Using chemical methods to covalently conjugate targeting ligands or polyethylene glycol (PEG) to the exosome surface. PEGylation prolongs the circulation half-life of exosomes in vivo and reduces clearance by the RES. In vivo imaging allows for the direct comparison of exosomal signal differences in the liver and spleen before and after modification, thereby quantifying the effect of PEGylation on half-life.

Verification of functional delivery: A standalone in vivo imaging signal is insufficient to prove functional delivery. When verifying "enhanced targeting," one must simultaneously demonstrate: 1) **physical distribution differences** (imaging data); 2) **evidence of functional delivery** (e.g., changes in target gene expression within target organs, verifiable by qPCR or Western blot); 3) **rigorous control groups** (e.g., empty vectors, unmodified exosomes).

4.6 Common Methodological Limitations and Standardization Recommendations

In in vivo exosome imaging research, common methodological limitations exist that can lead to erroneous conclusions if not avoided.

4.6.1 Common Limitations (and Mitigation Measures)

Dye-related false positives: Lipophilic dyes (e.g., PKH, DiR) easily form micelles or bind to plasma proteins, and are rapidly cleared by the RES, such as the liver and spleen. This can generate false-positive signals that mimic exosome distribution.

Mitigation measures: Dye-only controls and density gradient ultracentrifugation must be performed to exclude the effects of free dye.

Misleading labeling coverage: Genetic labeling may only mark a subpopulation of the total exosome population.

Mitigation measures: NTA and Western blot should be combined to confirm the labeling coverage ratio, with cross-validation against exogenous labeling methods.

Substrate distribution affecting BLI readings: Bioluminescence imaging relies on the distribution of substrates (e.g., D-luciferin or furimazine) in vivo. Differences in substrate accessibility across the blood-brain barrier, hepatic metabolism, and other processes may result in discrepancies between signal intensity in different tissues and the actual distribution of exosomes.

Mitigation measures: Evaluate the pharmacokinetic profiles of substrates during experimental design; if necessary, enhance signal stability through repeated administration or modified substrates.

4.6.2 Bias in Data Interpretation

Exosome signal accumulation $\neq$ functional delivery. The optical signal observed via in vivo imaging may originate from exosome membrane fragments or dye transfer, rather than the entry of intact exosomes into target cells. **Mitigation measures:** Findings must be corroborated by histological validation (e.g., immunofluorescence colocalization, RNA in situ hybridization) and functional readouts (e.g., gene expression changes, signaling pathway activation).

4.6.3 Inter-Experimental Variation and Reproducibility

Differences in isolation methods, labeling techniques, and dosage designs across various laboratories result in a lack of comparability in findings. **Mitigation measures:** Follow MISEV2018 guidelines by reporting particle count, protein content, and the particle-to-protein ratio; disclose raw images and ROI files to enhance data transparency.

4.7 Case Studies

Tumor Metastasis and Microenvironment

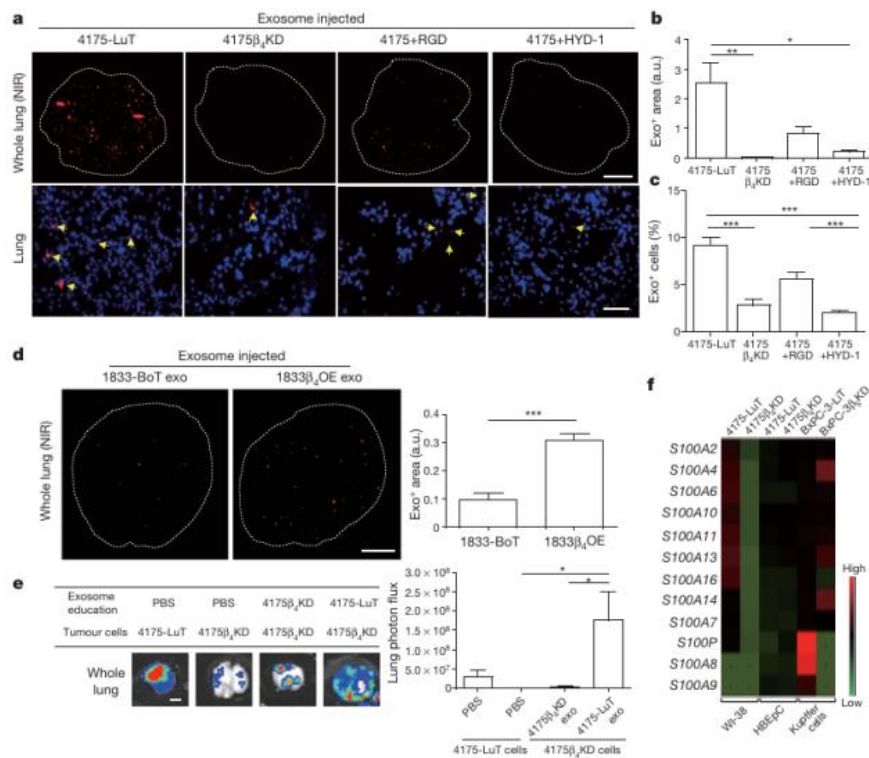


Figure 11 Study on Organotropic Exosome Metastasis

Since Stephen Paget proposed the metastatic organotropism hypothesis in 1889, metastatic organotropism has remained one of the greatest mysteries of cancer. Here, we demonstrate that exosomes from lung-tropic, liver-tropic, and brain-tropic tumor cells in mice and humans preferentially fuse with resident cells, specifically lung fibroblasts and epithelial cells, liver Kupffer cells, and brain endothelial cells. We demonstrated that tumor-derived exosomes are taken up by organ-specific cells, forming a pre-metastatic niche. Treatment of models with lung-tropic exosomes redirected the metastasis of bone-tropic tumor cells. Exosome proteomics has revealed distinct integrin expression patterns, and imaging studies have, for the first time, confirmed the pre-metastatic ecology theory of "exosomes lead, tumor cells follow" at a dynamic level^[5].

Immunotherapy

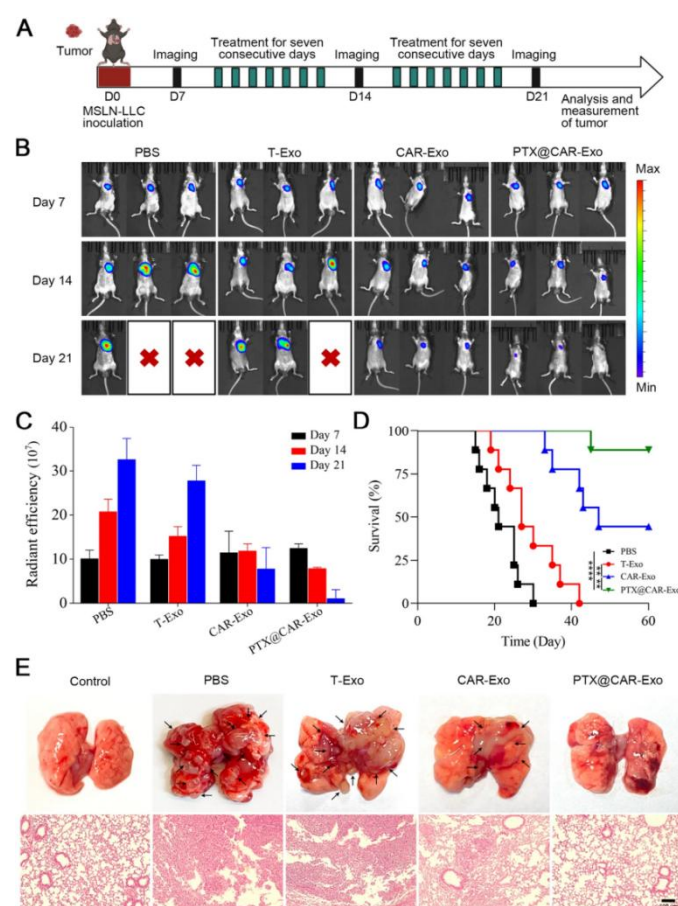


Figure 12 Combination of Exosomes and Immunotherapy

CAR-T cell exosomes carry functional TCR/CD3, which can accumulate locally in tumors and exert effector functions, while avoiding cytokine storms. In vivo imaging verified their localization and retention in tumor tissues. Chemotherapeutic drugs such as paclitaxel (PTX) have been widely used clinically, but they often cause multi-organ damage, such as liver and kidney dysfunction, due to systemic toxicity resulting from non-specific circulation. Therefore, it is necessary to develop new strategies to enhance the targeted antitumor effect of PTX and reduce systemic toxicity. CAR-Exos is a highly effective approach: firstly, its diameter is only 30–150 nm and it possesses hydrophobicity, allowing it to easily penetrate the tumor physical barrier to deliver PTX into tumor cells; secondly, the engineered CAR can recognize tumor-associated antigens, avoiding the cytotoxicity of PTX to normal tissues; finally, the targeted delivery characteristic can improve the bioavailability of PTX. Therefore, CAR-Exos holds promise as a biocompatible nanoplatform to minimize systemic toxicity in solid tumor therapy^[6].

Drug delivery

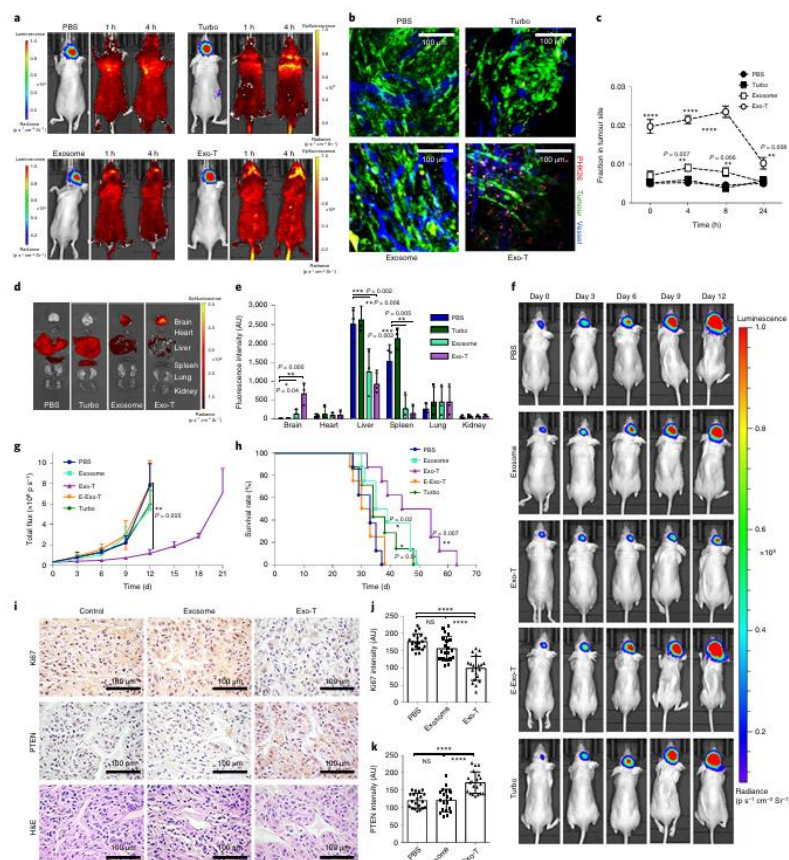


Figure 13 Exosomes Combined with Drug Delivery Systems Effectively Inhibit Tumor Growth

Due to their favorable pharmacokinetics and immunological properties, as well as their ability to penetrate physiological barriers that synthetic drug delivery vehicles cannot, exosomes have received significant attention as nucleic acid delivery vehicles. This study reports a cellular nanoporation method for the large-scale production of exosomes containing therapeutic mRNA and targeting peptides. By transfecting various source cells with plasmid DNA and employing local transient electrical stimulation, the release of exosomes carrying transcribed mRNA and targeting peptides is promoted. Compared with bulk electroporation and other exosome production strategies, the cellular nanoporation method increases the quantity of exosomes by more than 50-fold and the number of exosomal mRNA transcripts by over 1000-fold—even for cells with low baseline exosome secretion levels. In an orthotopic mouse model of PTEN-deficient glioma, mRNA-loaded exosomes successfully restored tumor suppressive function,

significantly enhancing tumor growth inhibition and extending survival. Cellular nanoporation technology holds the potential to make exosomes universal nucleic acid delivery vehicles, applicable in therapeutic areas requiring transcriptional manipulation^[7].

Chapter 5 Application of In Vivo Imaging in Immune System Research

The application of small animal in vivo optical imaging systems in immunology primarily focuses on three areas: immune disease monitoring, dynamic tracking of immune cells, and research into immune mechanisms. Detailed experimental procedures and typical case studies are provided below:

5.1 Technical Principles and Common Labeling Strategies

Bioluminescence (BLI)

Introduce the luciferase gene (Fluc, Rluc, Nluc, etc.) into target immune cells via transgenic or viral vectors; photons are produced following the injection of substrates (D-luciferin, coelenterazine), resulting in a high signal-to-noise ratio.

Fluorescence Imaging (FLI)

Utilize GFP, mCherry, Cy7, quantum dots, or near-infrared dyes to label cells/antibodies; tissue autofluorescence and hemoglobin absorption must be considered, with the 650–900 nm range typically selected.

Multimodal Fusion

BLI/FLI + X-ray/CT enables overlaying anatomical structures; BLI/FLI + high-frequency ultrasound allows for simultaneous evaluation of blood flow and immune cell migration.

5.2 Basic Experimental Procedures

5.2.1 Immune Disease Monitoring (using inflammatory diseases as an example)

Immune disease monitoring can be divided into the following three steps:

Probe Design and Injection

Use functional probes (such as ROS-responsive HA-AuNPs) to label disease-specific molecules. For example: Intravenous injection of HA-AuNPs probes (hyaluronic acid surface-conjugated with fluorescent dyes), which emit fluorescence upon cleavage by ROS.

Disease Model Establishment

Establish animal models (such as mouse arthritis models) to simulate disease states via chemical induction or genetic modification.

In Vivo Imaging and Data Acquisition

Following probe injection, utilize a small animal in vivo optical imaging system at various time points (e.g., 0 h, 24 h, 48 h) to capture bioluminescence or fluorescence signals for quantitative analysis of disease progression.

Taking inflammatory diseases as an example: *Candida albicans* is a major pathogenic fungus that employs various metabolic adaptations to evade immune cells and cause systemic disease. Here, we describe a protocol for measuring one of these adaptations—the ability to survive in a hypoxic microenvironment. Following the successful establishment of a mouse subcutaneous infection model, hypoxia is induced by *C. albicans*. The hypoxic conditions are measured using the HypoxiSense 680 fluorescent dye. This dye enables visualization of hypoxic regions, helping researchers assess tissue oxygenation. Finally, quantitative analysis was performed on the resulting in vivo fluorescence imaging data.

Experimental Procedure:

(1) Fungal culture and mouse preparation (Days 1–3)

- 1) Using an inoculating loop, inoculate the frozen stock of *C. albicans* into 10 mL of SC medium in a 50 mL tube; incubate with shaking at 180 rpm at 30 °C for 20 hours.
- 2) Take 10 µL of the *C. albicans* culture prepared the previous day and add it to 10 mL of fresh SC medium in a 50 mL tube. Incubate the subculture under the same conditions.
- 3) On day 3, count the cells using a cell counter and prepare a fresh subculture in 10 mL of SC medium at an initial density of 5×10^7 cells/mL; incubate for 3 hours. Centrifuge at $3,000 \times g$ for 10 minutes at room temperature, then resuspend the fungi in 1 mL of 1x PBS and count the fungal cells using a hemocytometer.
- 4) Adjust the fungal concentration to 5×10^8 cells/mL, and store on ice if immediate use is required. Calculation example: To prepare 1 mL of yeast suspension at a concentration of 5×10^8 cells/mL from a stock suspension of 8×10^8 cells/mL, add 625 µL of the stock suspension to 375 µL of PBS.
- 5) All mice (B6(Cg)-Tyrc-2J) were injected intraperitoneally with 100 µL of HypoxiSense 680 dye (2 nmol/100 µL per mouse) 24 hours prior to subcutaneous infection with *C. albicans*.

(2) Subcutaneous infection (Day 4)

- 1) Place the mice into the anesthesia chamber and adjust the isoflurane gas concentration to 2.5% in the gas anesthesia system. Remove the mice from the anesthesia chamber and gently place their noses into the isoflurane nose cone.
- 2) After anesthetizing the mouse, use a shaver to remove the hair from the dorsal or ventral region (Figure 1).

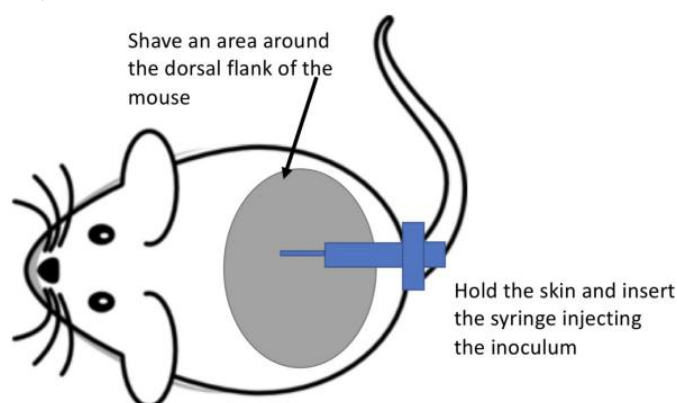


Figure 14 Schematic of Subcutaneous Infection Procedure

- 3) Lift the mouse skin with forceps. Insert the syringe into the lifted skin and infect the mouse with the prepared 100 μL *C. albicans* inoculum (5×10^7 cells). A small bleb will be visible under the animal's skin for several minutes.
- 4) Inject control group animals in a similar manner with 100 μL of 1x PBS.
- 5) Place the animals under a heat lamp until they have fully recovered from anesthesia.

(3) In vivo imaging (Days 4–7)

- 1) Anesthetize the mice with 2.5% isoflurane in an induction chamber, then transfer them to the imaging manifold, ensuring each animal's nose is positioned within a nose cone.
- 2) Select filter settings appropriate for the dye's emission and excitation wavelengths (675 ex/720 em).
- 3) Perform time-zero image acquisition for both groups of animals on day 4 post-initial infection (Figure 2). Capture in vivo fluorescence generated by HypoxiSense dye activity as a marker for hypoxia, using an exposure time of 0.5 seconds.

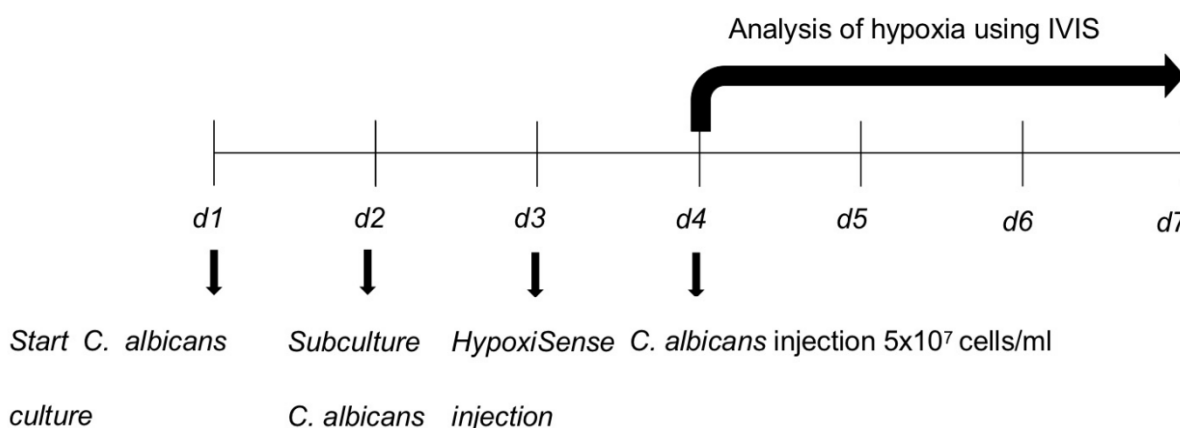


Figure 15 Schematic of Experimental Procedure

Data analysis:

- (1) Draw a region of interest (ROI) in the area corresponding to the abscess, covering the region of high fluorescence. Place a background ROI in a dark area to define the background.
- (2) Subtract the background fluorescence value from the fluorescence value of each animal.
- (3) Plot the data with the fluorescence value on the y-axis and the group on the x-axis. Use a two-tailed Mann-Whitney U-test for statistical significance testing between the infected group and the control group.

5.2.2 Dynamic Tracking of Immune Cells

Innate immune cells include leukocytes such as natural killer (NK) cells, dendritic cells (DCs), cytotoxic T lymphocytes (CTLs), regulatory T lymphocytes, neutrophils, monocytes, eosinophils, macrophages, and basophils. Among these, NK cells and T cells, acting as cytotoxic lymphocytes, can specifically recognize cancer cells through various immune receptors and serve as promising and effective immunotherapeutic agents for multiple cancers. To date, NK cells and T cells have been widely used in preclinical research and clinical practice to treat different types of cancer, such as acute myeloid leukemia, ovarian cancer, breast cancer, pancreatic cancer, hepatocellular carcinoma, and glioblastoma. The efficacy of immunotherapy depends directly on the in vivo survival and persistence of the transplanted NK and T cells. Therefore, tracking and

monitoring the viability of these immune cells in vivo is of great significance for evaluating therapeutic effects during immunotherapy.

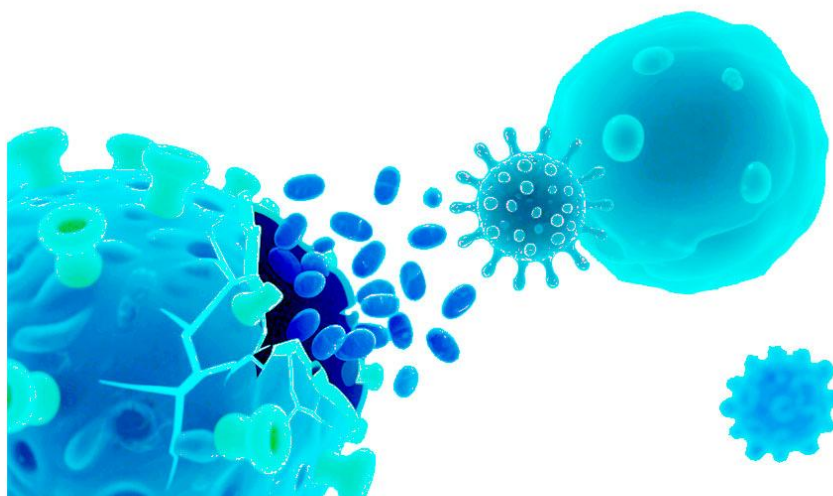


Figure 16 Schematic of dynamic immune cell tracking

5.2.2.1 Procedures for dynamic immune cell tracking

(1) Cell labeling

Genetic labeling: Transfect T cells with a luciferase gene (e.g., Fluc) using a lentiviral vector, or isolate immune cells from transgenic bioluminescent mice (e.g., Luc⁺ mice).

Dye Labeling: Directly label monocytes or T cells in vitro using near-infrared fluorescent dyes (e.g., DiD).

(2) Cell Transplantation and Model Establishment: Transfer the labeled cells to recipient animals (e.g., tumor model mice) via tail vein or local injection.

(3) Real-time Imaging and Migration Analysis: Perform imaging at regular intervals to record the distribution of cells in lymph nodes, the spleen, or tumor sites, and analyze migration pathways and functional status (proliferation, activation).

(4) Functional validation: Validate imaging results using ex vivo analysis (e.g., flow cytometry, frozen sections).

5.2.2.2 Case study analysis

Taking Upconversion Nanoparticle (UCNP) labeling of NK cells as an example:

(1) Synthesis and Surface Modification of UCNPs (Water-solubility transformation)

Synthesis of core UCNPs ($\text{NaYF}_4:\text{Yb}^{3+}/\text{Tm}^{3+}$)

- 1) Dehydration: Mix rare earth salts, oleic acid, and 1-octadecene, and heat to 150 °C.
- 2) Injection of Fluorine and Alkali Sources: Rapidly inject NaOH and NH_4F solutions, and incubate at 50 °C for 30 minutes.
- 3) High-temperature crystallization: Heat to 290 °C and react for 2 hours under argon protection.
- 4) Purification: After cooling, add ethanol and centrifuge to collect precipitate; repeat 3 times and finally disperse in cyclohexane.

(2) Cell culture and labeling (electroporation)

- 1) Cell preparation: Collect 1×10^7 NK-92 cells and resuspend in 100 μL of culture medium.
- 2) Addition of UCNPs: Mix with carboxyl-PEG-UCNPs (final concentration approximately 50 $\mu\text{g}/\text{mL}$).
- 3) Electroporation: Apply electric pulse using a preset program (e.g., OPT2).
- 4) Recovery culture: Incubate at 37 °C for 20 minutes → dilute to 2 mL of culture medium → continue incubation for 1–2 hours.
- 5) Washing: Wash 3 times with PBS to remove free UCNPs and dead cells.

(3) Cell function validation assays

1) Cell viability assay

Method: Trypan blue staining.

Result: Calculate cell viability.

2) Cytokine secretion ($\text{IFN-}\gamma$, $\text{TNF-}\alpha$)

ELISA:

Collect the supernatant 24 hours after electroporation.

Quantify using human $\text{IFN-}\gamma/\text{TNF-}\alpha$ ELISA kits (R&D Systems).

Compare differences between the labeled and untreated groups.

3) Cytotoxic function (against K562 target cells)

Calcein-AM release assay:

Target cells (K562) are pre-labeled with Calcein-AM.

Co-culture for 4 hours at an effector-to-target ratio ($\text{E:T} = 5:1$ or $1:1$).

Measure fluorescence intensity and calculate the lysis rate.

Compare the killing efficacy between the labeled and control groups.

(4) Imaging and distribution experiments

1) In vivo sensitivity test (subcutaneous injection)

Cell titration: 1.25×10^4 – 1×10^6 cells/50 μ L PBS

Injection site: Subcutaneous (abdominal)

Imaging time points: Day 0 and Day 1

Results: Perform linear regression of signal intensity against cell number and calculate the R^2 value.

2) In vivo biodistribution tracking (intravenous injection)

Dosage: 2×10^7 cells/200 μ L PBS

Time points: 0.5, 1, 3, 6, and 24 hours

Imaging Mode: Whole-body fluorescence imaging + Ex vivo organ imaging (lung, liver, spleen)

Tissue Validation: Frozen sectioning → Fluorescence colocalization

5.2.3 Investigation of Immune Mechanisms (Transgenic Animal Models)

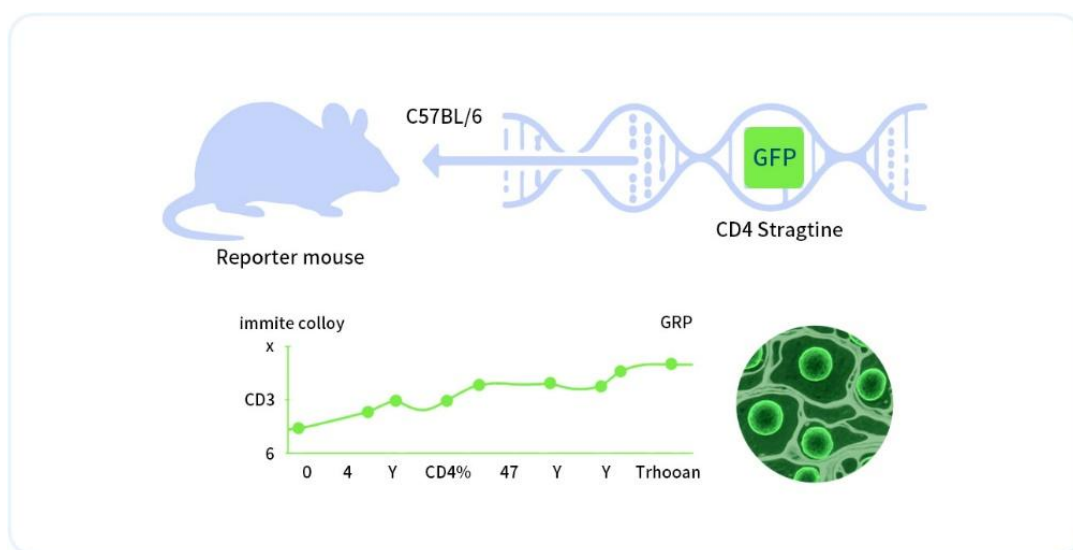


Figure 17 Schematic of Transgenic Animal Model Establishment

The primary workflow for immune mechanism research is as follows:

- (1) Generation of transgenic animals: Use inflammation-related gene promoters (e.g., NF- κ B, iNOS) to drive luciferase expression to generate transgenic mice (e.g., Tg(NF κ B-RE-luc)).
- (2) Induction of immune response: Inject an immunostimulant (such as LPS to induce inflammation) or a pathogen (such as a virus).
- (3) Gene expression monitoring: Use in vivo imaging to observe luciferase signals, reflecting the spatiotemporal expression dynamics of the target gene.
- (4) Mechanistic intervention: Administer gene knockout or drug treatments and compare signal changes to explore molecular mechanisms (e.g., caffeine inhibiting NF- κ B expression).

Classification and characteristics of transgenic animal models commonly used in immune mechanism research:

(1) Overexpression model

IL34 transgenic mice: Overexpression of the human IL34 gene in C57BL/6J mice, used to study macrophage differentiation and splenic immune regulation. The model exhibits splenic white pulp hyperplasia and enhanced immune cell activity.

HLA-G transgenic mice: Lentiviral vector-mediated expression of the human HLA-G gene in FVB/N mice, used for transplantation immunology research. Gene integration is achieved via embryonic microinjection.

TNFRSF6B transgenic lupus model: Human β -actin promoter-driven TNFRSF6B expression leads to lymphadenopathy, antinuclear antibodies, and glomerulonephritis in female mice, mimicking systemic lupus erythematosus (SLE).

(2) Gene knockout/knock-in models

Fc γ RIIB knockout SLE model (B6;129S-Fcgr2btm1Ttk/J): Deficiency in this inhibitory receptor leads to impaired immune complex clearance, triggering nephritis and elevated autoantibodies.

CD45E613R knock-in model: Glutamic acid to arginine mutation at position 613 of the CD45 gene causes abnormal T/B cell activation, lymphoproliferation, and autoimmune nephritis.

B cell-specific Ewsr1 knockout model: Utilizing the Cre/loxP system (driven by Mb1-Cre) to knock out the Ewsr1 gene in B cells, enhancing humoral immunity and antibody production, suitable for monoclonal antibody screening.

(3) Humanized immune system model

Modified BLT model:

Procedure: Implantation of human thymic tissue into NSG mice, injection of liver-derived CD34⁺ cells, and delivery of cytokines (IL-3/IL-7/GM-CSF) via AAV8 vectors.

Advantages: Long-term stable reconstitution of multilineage immune cells (30 weeks), supports HLA-restricted T cell development; used for cancer immunotherapy research.

(4) Inducible expression model

Cre/loxP-regulated HBsAg model: HBsAg gene expression is activated by Cre recombinase to avoid immune tolerance. Achieved via microinjection or hydrodynamic tail vein injection of naked plasmids, used for HBV-related immune injury research.

The following table summarizes the characteristics of major immune-related transgenic animal models:

Table 3 Characteristics of Major Immune-Related Transgenic Animal Models

Model Type	Representative Model	Immunophenotype	Applications
Overexpression	IL34 transgenic mouse	Splenic white pulp hyperplasia, enhanced macrophage differentiation	Innate immunity regulatory mechanisms
Gene knockout	FcγRIIB ^{-/-}	Elevated autoantibodies, glomerulonephritis	Etiology of SLE
Humanized	Modified BLT model	Multilineage immune reconstitution, HLA-restricted T cells	Cancer immunotherapy
Inducible expression	Cre/loxP-HBsAg	Acute hepatitis, anti-HBsAg antibody production	HBV immunopathology

5.2.4 Immunodeficient Mouse Models

Immunodeficient models are animal models with deficiencies in one or more components of the immune system. With the continuous development of genetic tractability technology, an increasing number of immunodeficient mouse strains have been developed, playing an increasingly vital role in research fields such as immunology, infectious diseases, oncology, and stem cell biology.

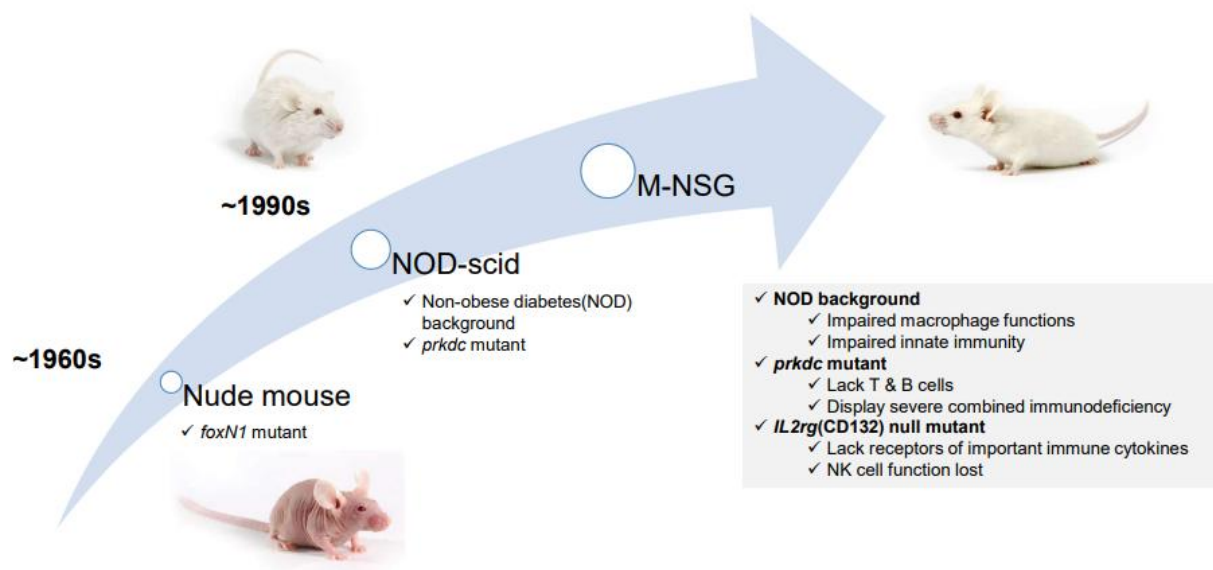


Figure 18: Development History of Transgenic Animal Models

The following classification of common immunodeficient mouse models and their core characteristics is ordered by the degree of immunodeficiency (low to high), incorporating application fields and recent advancements:

5.2.4.1 Basic Immunodeficient Models

(1) Nude mice

Genetic background: *Homozygous Foxn1* mutation.

Deficiency characteristics: Hairless, athymic, complete T-cell deficiency; normal B-cell and NK-cell function.

Applications: Nude mice are ideal hosts for rapidly growing tumor cell lines and are widely used in solid tumor xenografts (CDX models), immunology, and infectious disease research.

Limitations: Residual NK cell activity may lead to graft rejection; shorter lifespan (susceptible to hepatitis).

(2) SCID mice

Genetic background: *Homozygous Prkdc* mutation (DNA repair enzyme deficiency).

Deficiency characteristics: Combined T and B cell deficiency, but NK cells and macrophages are normal; approximately 20% of mice exhibit "leakiness" (partial recovery of immune function).

Applications: Suitable for syngeneic and xenogeneic tumor transplantation; can be used for hematological malignancy PDX models and immune reconstitution research.

5.2.4.2 Moderate Immunodeficiency Models

(1) NOD-SCID Mice

Genetic background: A cross between the NOD (non-obese diabetic) strain and SCID, carrying NOD innate immune deficiencies (low complement activity, impaired macrophage function).

Deficiency characteristics: T/B cell deficiency, significantly reduced NK cell activity (more profound than SCID), no diabetic phenotype, lack of hemolytic complement activity, and bone marrow developmental defects.

Applications: Hematopoietic stem cell transplantation, various tumor models; however, prone to lymphoma (lifespan ≤ 8 months), making them unsuitable for long-term transplantation experiments.

5.2.4.3 Severe Immunodeficiency Models (Triple Gene Deficiency)

Knocking out the *IL2rg* gene (cytokine receptor common gamma chain) in the NOD-scid background further eliminates NK cell function, significantly improving the efficiency of human cell transplantation:

(1) B-NSG/B-NDG mice

Genetic background: NOD-SCID + IL2rg knockout.

Deficiency characteristics: Complete absence of T/B/NK cells, weak macrophage phagocytosis, and no leakiness.

Applications: Lifespan up to 1.5 years, supports tumorigenesis from minimal cell numbers, suitable for HSC, PBMC, and PDX transplantation. Humanized immune system reconstitution, CAR-T efficacy evaluation.

(2) C-NKG mice

Genetic background: NOD-SCID + IL2rg knockout (similar to B-NSG).

Characteristics: Extremely low incidence of lymphoma, supports neonatal mouse HSC transplantation (higher T-cell reconstitution efficiency).

Applications: HIV infection models, orthotopic transplantation of solid tumors (such as pancreatic cancer, lung cancer).

(3) NCG mice

Genetic background: NOD + Prkdc/IL2rg double knockout.

Application: Lifespan >89 weeks; widely used for PDX efficacy testing (e.g., evaluation of the lung cancer targeted drug erlotinib).

(4) BRGSF mice

Genetic background: Rag2/IL2rg/Sirpα/Flk2 quadruple gene modification.

Characteristics: Intact complement system, inhibited macrophage phagocytosis; suitable for studying complement-dependent therapies (e.g., antibody drugs).

Application: HIV cellular immunotherapy, short-term human PBMC experiments.

Table 4 Comparison of Major Immunodeficient Mouse Strains

Strain	Immunodeficiency characteristics	Application fields	Advantages and limitations
Nude	T-cell deficiency; normal B/NK cells	Solid tumor xenografts, immunology	Low cost; NK activity limits graft survival
SCID	T/B cell deficiency, normal NK cells	Hematological malignancy PDX models	Prone to immune "leakage"
NOD-SCID	T/B cell deficiency + reduced NK activity	Stem cell transplantation, short-term oncology studies	Short lifespan (≤ 8 months), prone to lymphoma
B-NSG	Complete T/B/NK cell deficiency	Humanized models, tumorigenesis from minimal cell numbers	Long lifespan (1.5 years), no leakage
C-NKG	T/B/NK deficiency + low lymphoma risk	HIV models, orthotopic tumors	High T-cell reconstitution efficiency in neonatal transplants
BRGSF	T/B/NK deficiency + intact complement system	Complement-dependent therapies, PBMC studies	Suitable for antibody drug assessment

B-NDG[®] mice

B-NDG[®] mice (NOD-Prkdcscid IL2rgtm1/Bcgen) are IL2rg knockout mice on a NOD-scid genetic background. Lacking mature T, B, and NK cells, they are internationally recognized as highly immunodeficient models ideal for human cell or tissue transplantation.

Featuring the NOD scid-IL2rg knockout background, B-NDG[®] mice exhibit a severe immunodeficiency phenotype characterized by a lack of mature T, B, and functional NK cells, as well as impaired macrophage and dendritic cell function. They are highly suitable for human hematopoietic stem cell (CD34⁺ HSC) and peripheral blood

mononuclear cell (PBMC) transplantation to generate humanized mice with a human immune system.

Human immune system reconstruction in B-NDG mice via human CD34⁺ HSC transplantation and efficacy evaluation of anti-human PD-1 antibodies:

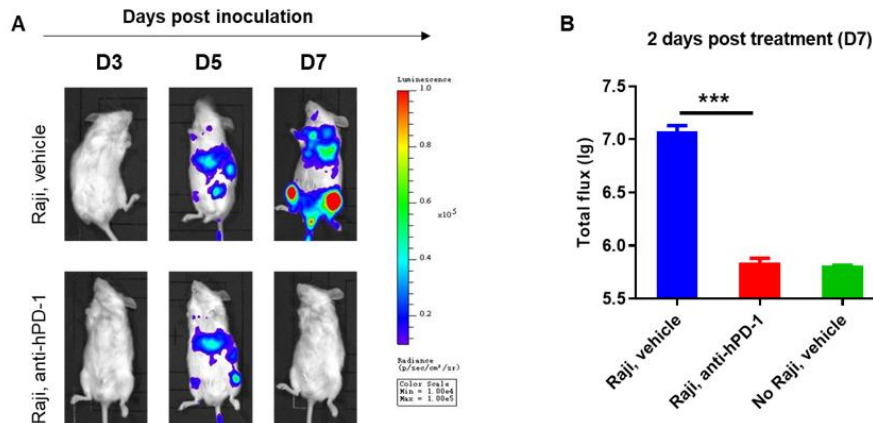


Figure 19 Monitoring of Lymphoma Xenograft Model

B-NDG mice reconstituted with CD34⁺ hematopoietic stem cells are used for drug efficacy evaluation. Human lymphoma cell line B-luciferase-GFP Raji cells (5E5) were inoculated via tail vein into adult B-NDG mice reconstituted with human CD34⁺ hematopoietic stem cells. Mice were treated with anti-human PD-1 antibody 5 days after tumor cell inoculation. A significant inhibitory effect of the anti-human PD-1 antibody on tumor cell growth was observed on day 7. The results indicate that CDX tumor models established in HSC-reconstituted B-NDG mice provide a robust preclinical platform for in vivo antibody evaluation. Data are expressed as mean $\pm$ SEM.

5.3 Typical Case Studies

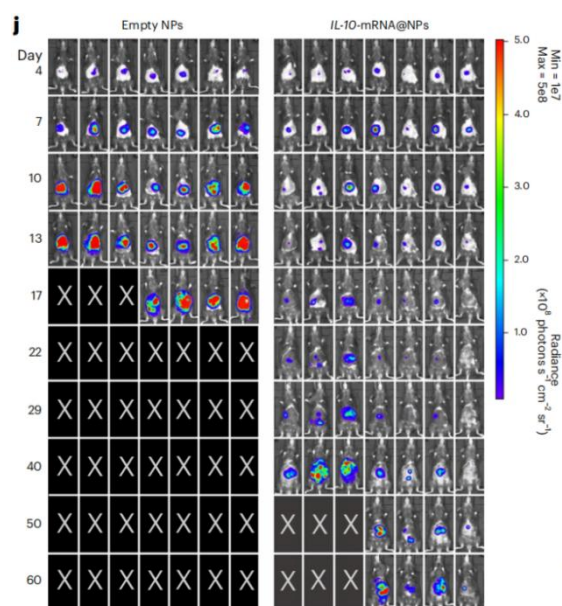


Figure 20. Efficacy Evaluation of IL-10-mRNA@NPs

Intravenous injection of IL-10 messenger RNA (mRNA) nanoparticles (IL-10-mRNA@NPs) can induce potent immunosurveillance in various preclinical tumor models while reducing systemic toxicity. In particular, IL-10-mRNA@NPs can continuously produce IL-10 within tumors, promote massive infiltration and proliferation of cytotoxic T cells, induce the activation and maturation of dendritic cells, and enhance the expression of major histocompatibility complex class I molecules in immunosuppressive early-stage orthotopic hepatocellular carcinoma. Furthermore, in a mouse model of advanced orthotopic hepatocellular carcinoma, the combination of IL-10-mRNA@NPs and an immune checkpoint inhibitor achieved complete tumor clearance in 43% of the mice and extended the median survival by 6-fold compared to the immune checkpoint inhibitor monotherapy group. More importantly, this combination strategy can induce durable antitumor immune memory, providing 100% protection against tumor rechallenge. The strategy of intravenous administration of IL-10-mRNA@NPs is expected to overcome the challenges faced by recombinant IL-10 in clinical trials and is applicable to the treatment of a wider range of immunosuppressive tumors^[8].

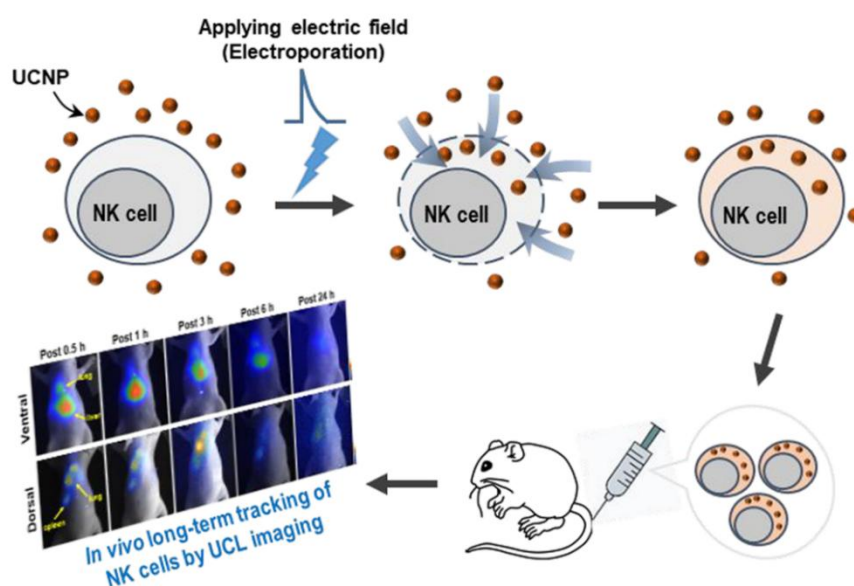


Figure 21. Efficacy Evaluation of IL-10-mRNA@NPs

Long-term in vivo tracking of NK cells labeled with UCNPs using electroporation (EP) technology. In this study, carboxyl-PEG modified $\text{Tm}^{3+}/\text{Yb}^{3+}$ -doped upconversion nanoparticles (UCNPs) were efficiently labeled into non-phagocytic NK-92 cells with low toxicity via electroporation, enabling high-sensitivity, background-free near-infrared upconversion luminescence (UCL) imaging of their in vivo distribution. Results showed that labeling did not affect NK cell viability or cytotoxicity. Following intravenous injection, cells initially accumulated in the lungs before migrating to the liver and spleen, providing a reliable tool for real-time monitoring of NK cell-based cancer immunotherapy.

Chapter 6 Recommendations for Imaging System Selection

In biomedical research, *in vivo* optical imaging systems, as a non-invasive tool, can monitor complex biological processes in experimental animals in real-time, providing significant support for scientific research. However, given the variety of small animal *in vivo* optical imaging systems and their complex technical parameters—such as resolution, dark current, F-number, and spectral unmixing capability—researchers often struggle to determine how these parameters impact experimental outcomes.

Which core metrics are truly decisive for experimental success? What kind of system design can ensure the reliability and reproducibility of data? Based on the practical requirements of scientific research, this chapter systematically reviews key performance parameters and equipment selection guidelines to help researchers make informed decisions and select the most appropriate imaging tools for their experimental projects.

6.1 Hardware Performance

In *in vivo* imaging, the signals to be detected are often extremely weak, placing high demands on the sensitivity of the imaging system. If the system's noise control is inadequate, these low-abundance signals can easily be overwhelmed by background noise, resulting in the loss of meaningful data. Therefore, when selecting an imaging system, one should look beyond nominal specifications and understand the underlying physical mechanisms and their practical implications.

6.1.1 CCD Cooling Depth and Dark Current

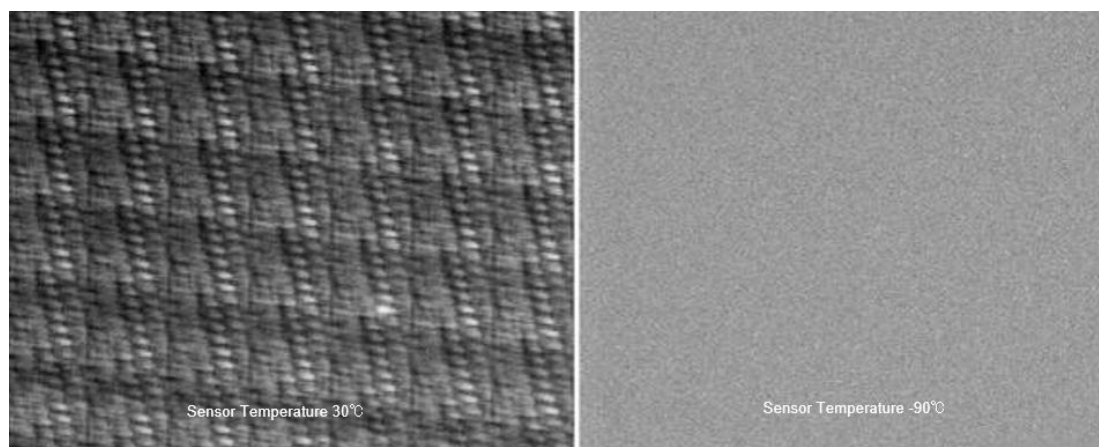


Figure 22 Effect of CCD camera temperature on dark current

When a CCD (charge-coupled device) is in operation, the substrate material generates a small amount of free electrons due to thermal excitation. These electrons mix with the signal electrons generated by photons, creating an indistinguishable "background noise," which is known as **dark current**. Deep cooling is the most effective method for reducing dark current; for every 6–8 °C reduction, the dark current is halved. Therefore, a system with deeper cooling provides a cleaner background and higher image quality during long exposures.

6.1.2 Readout Noise

When a CCD transfers the received photon signal (charge) out of the camera and converts it into a digital signal, this readout process itself generates an inherent electronic noise, which is known as readout noise. In in vivo imaging experiments involving low-light and weak fluorescence signals, readout noise becomes a decisive factor. Extremely low readout noise means the camera can detect weak signals more effectively, resulting in cleaner images and, consequently, higher-quality data.

6.1.3 Signal-to-Noise Ratio (SNR)

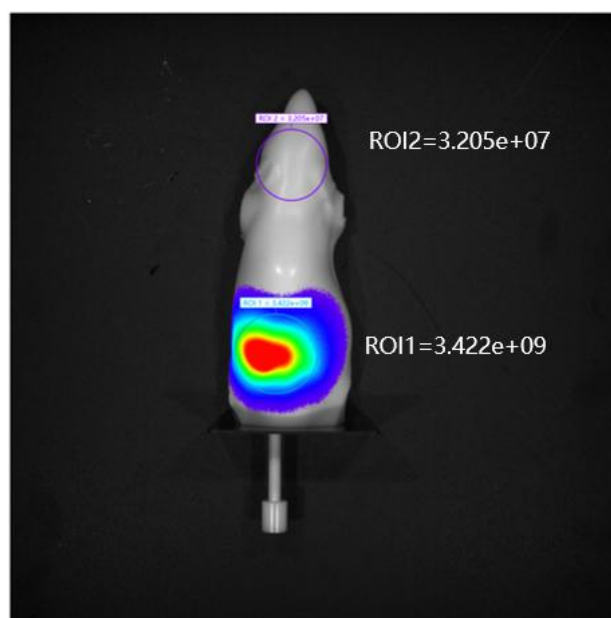


Figure 23 Comparison of Signal-to-Noise Ratio

The signal-to-noise ratio is the ultimate core metric for measuring system performance. It is a ratio where the numerator is the effective signal and the denominator represents various noise sources (including dark current, readout noise, and stray fluorescence). An excellent system achieves the highest signal-to-noise ratio by minimizing the noise terms in the denominator while maximizing the signal term in the numerator.

6.1.4 CCD Pixels

Higher pixel count = higher resolution: The total pixel count of a camera refers to the number of photosites on its sensor. The more pixels there are, the more finely the sensor can divide the scene and the more detail it can capture.

The total number of pixels is important, but the physical size of individual pixels is even more important.

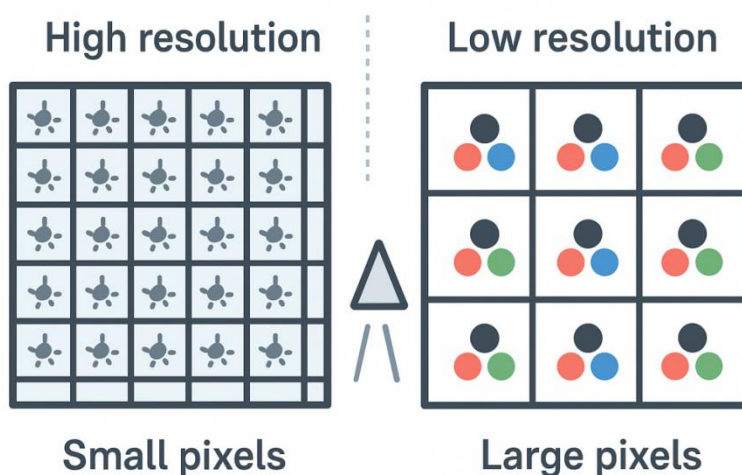


Figure 24 Schematic Comparison of Large Pixels and Small Pixels

Large pixels vs. small pixels:

Larger pixels: For a fixed sensor size, a lower pixel count means a larger light-sensitive area per pixel. Advantages: Captures more light and offers a superior signal-to-noise ratio. In photographs, this manifests as less noise and clearer image quality, especially in low-light environments (such as night scenes or indoors), where it performs significantly better than small sensors with high pixel counts. The dynamic range (the ability to record both the brightest and darkest details simultaneously) is also generally better.

Smaller pixels: When more pixels are packed onto a sensor of the same size, the light-sensitive area of each pixel becomes smaller. Disadvantages: More prone to thermal and electronic noise; image quality degrades significantly at high ISO (sensitivity) levels.

A vivid analogy:

Imagine a CCD sensor as a swimming pool, and the pixels as cups used to catch raindrops.

High pixel count (small pixels): Filling the swimming pool with many small cups (pixels). With more cups, the position where raindrops fall can be recorded more precisely (high resolution), but each cup collects less water (light).

Low pixel count (large pixels): Filling the swimming pool with a few large cups. With fewer cups, the recorded positions are less precise (low resolution), but each cup can collect more water (light), making it easier to measure (better signal-to-noise ratio).

6.2 Optical Design

Optical design is a core component of imaging systems, and its performance directly determines the efficiency with which the system captures photons. High-quality optical components can significantly enhance signal acquisition, exerting a decisive impact on imaging speed and the signal-to-noise ratio of the final image.

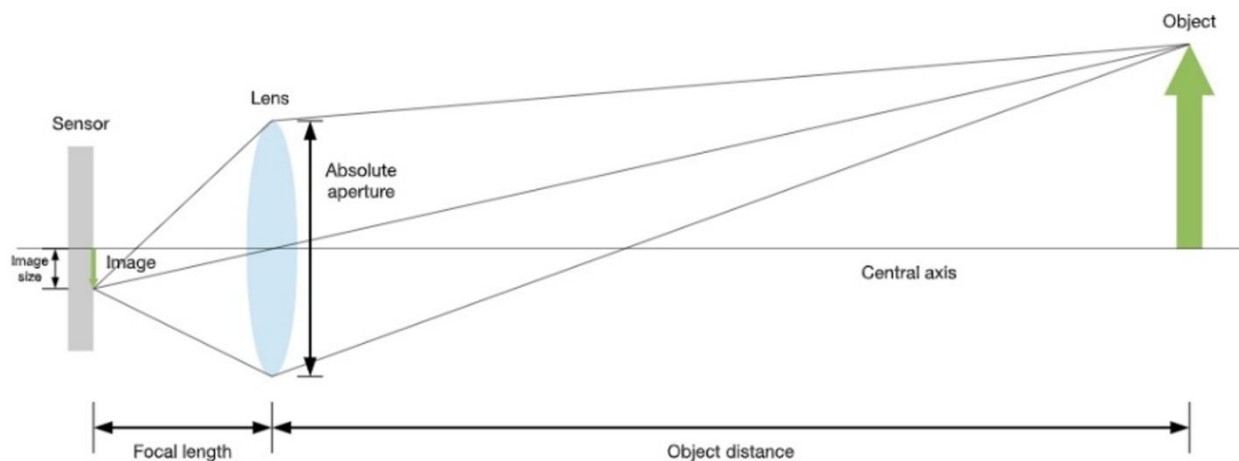


Figure 25 Schematic of Optical Design

6.2.1 F-number and Large-Aperture Lenses

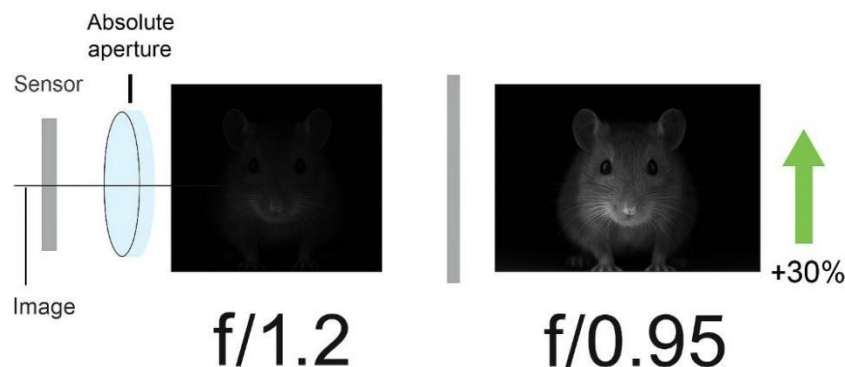


Figure 26 Impact of F-number on Imaging Results

F-number: Defined as the ratio of the lens's focal length (f) to the entrance pupil diameter (D), i.e., $F = f / D$.

Physical significance: The F-number is a relative value representing the lens's light-gathering efficiency rather than an absolute measure. The smaller the F-number, the larger the aperture opening, allowing more light to enter the camera per unit of time.

In vivo imaging, especially fluorescence imaging, is always battling an enemy: extremely low photon signals. This is because:

Fluorescence signals within biological organisms are extremely weak.

Biological tissues have strong absorption and scattering effects on light.

To reduce phototoxicity and photobleaching, it is necessary to limit excitation light intensity and exposure time.

Therefore, maximizing the collection of every precious photon is crucial. This is where the role of a large-aperture lens (low F-number) comes into play:

Significantly improve the signal-to-noise ratio (SNR)

Signal: A large aperture allows more (true) fluorescence photons from the sample to reach the detector.

Noise: Camera readout noise and dark current noise are fixed and do not vary with the amount of incident light.

Result: Signal-to-noise ratio (SNR) $\approx \sqrt{\text{Signal}}$. A 4-fold increase in light collection theoretically yields a 2-fold improvement in SNR. This results in cleaner images with darker backgrounds and sharper targets, facilitating quantitative analysis.

Significant reduction in exposure time

Increased light collection allows for the capture of sufficiently bright images in less time.

Benefits:

Reduced motion artifacts: Breathing and minor movements are inevitable in live animals; shorter exposure times can "freeze" motion, resulting in sharper images.

Reduced phototoxicity and photobleaching: Shortening exposure to lasers or illumination better preserves sample viability, which is critical for long-term dynamic observation.

Increased throughput: In high-throughput screening, shorter exposure times translate to faster imaging speeds.

Allows the use of lower doses of probes or weaker excitation light

With a fixed exposure time, the high light collection efficiency of a large aperture means that lower concentrations of fluorescent probes or weaker intensity excitation light can be used to achieve the same image brightness.

Benefits:

Reduced toxicity: Minimizing the potential toxic side effects of probes and excitation light on animals ensures that experimental data more closely reflect physiological states.

Extended observation time: Reducing photobleaching effects enables long-term observation.

6.2.2 Excitation Light Source and Spectral Continuity

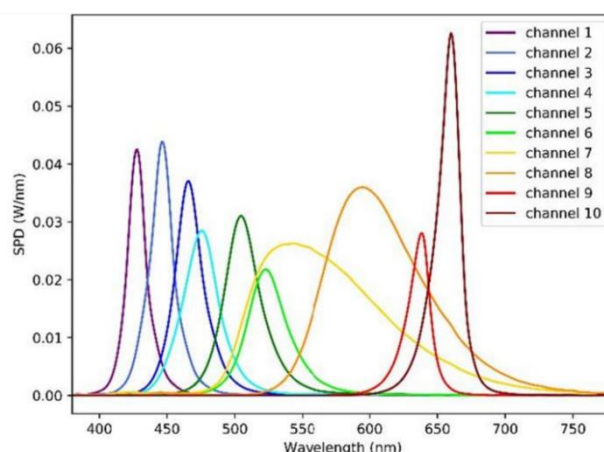


Figure 27 Excitation power of LED light sources

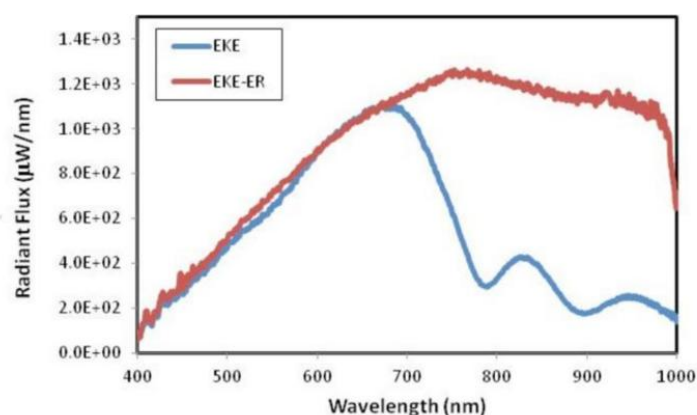


Figure 28 Excitation power of tungsten halogen light sources

Fluorescence experiments rely on excitation light. The quality of the light source directly affects the excitation efficiency. **Continuous-spectrum light** sources provide a continuous spectrum covering the entire visible and near-infrared range, better supporting multi-wavelength probe experiments and enabling the use of future novel probes.

Most in vivo imaging systems use **halogen lamps** as light sources for several key reasons: In vivo imaging, especially that based on bioluminescence and fluorescence, requires a powerful light source to excite fluorescent probes (e.g., GFP, RFP, or various dyes). Halogen lamps (especially high-power xenon lamps) offer unparalleled advantages in this regard:

Continuous and broad spectrum

Halogen/xenon lamps produce a continuous spectrum from the ultraviolet (~300 nm) to the near-infrared (~1000 nm and beyond), closely mimicking natural sunlight.

This is crucial because different fluorescent probes have different optimal excitation wavelengths (e.g., GFP is excited at approximately 488 nm, while RFP is at approximately 558 nm). A broad-spectrum light source can efficiently excite multiple different fluorescent labels; the desired excitation band is simply selected by changing the filter. A single device can meet a variety of experimental needs, offering high flexibility and cost-effectiveness.

Extremely high brightness (light intensity)

The subject of in vivo imaging is an entire living animal (e.g., a mouse), and light must penetrate tissues such as skin, muscle, and bone to reach deep-seated targets (e.g., tumors, organs) and be captured by the detector.

During this process, the light is scattered and absorbed, resulting in significant attenuation. Therefore, an extremely bright light source is required to ensure that sufficient photons reach the target and return to the camera to generate a valid signal. High-power xenon lamps (typically 100W or more) can provide this required high-intensity light.

Comparison with other light sources (Why not use LEDs?)

To better understand why xenon lamps are used, consider the limitations of other light sources:

LED (Light-Emitting Diodes)

Advantages: Energy-efficient, long lifespan, compact size, low heat generation, and instant switching.

Disadvantages:

Narrow spectrum: A single LED typically emits light only in a very narrow band (e.g., 470 nm $\pm$ 10 nm). To cover a broad spectrum, multiple LEDs of different wavelengths must be integrated, which increases the complexity and cost of the system.

Insufficient light intensity: Although LED technology is advancing rapidly, high-power xenon lamps still offer an advantage in total luminous flux when extremely high light intensity is required for penetrating thick tissues, especially for probes with lower excitation efficiency.

Current status: In recent years, high-power multi-wavelength LED light sources have been developing quickly and are beginning to replace halogen lamps in some applications where penetration depth requirements are not stringent. However, in high-end deep tissue imaging, halogen/xenon lamps remain the mainstream.

6.2.3 Optical Fiber Autofluorescence Control

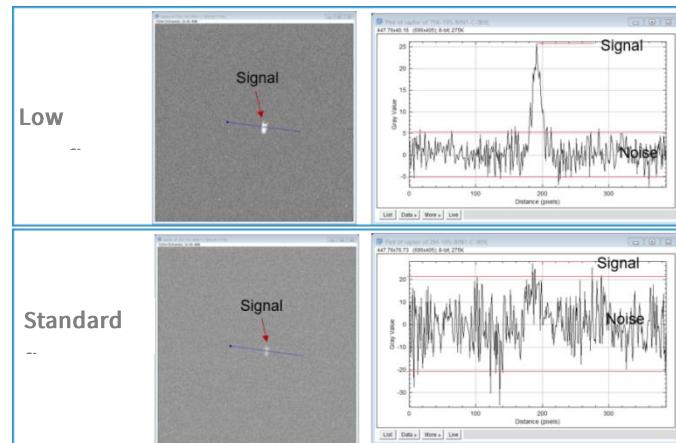


Figure 29 Comparison of Signal-to-Noise Ratios of Different Fluorescence Fibers Under the Same Light Source

During excitation light transmission, the optical fiber itself emits weak fluorescence, interfering with the target signal. Using **ultra-low fluorescence** fibers reduces background interference at the source, ensuring high signal purity.

Problem (defects of standard optical fibers): When standard optical fibers are irradiated by excitation light (especially high-energy UV or blue light), they emit a weak fluorescence themselves (referred to as "autofluorescence" or "fiber fluorescence"). This fluorescence becomes background noise.

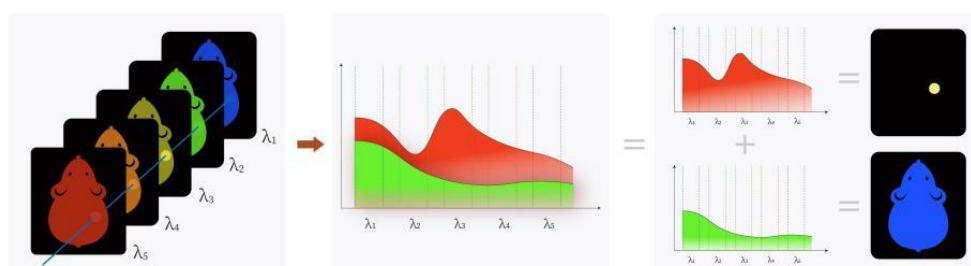
Impact on in vivo imaging: Fluorescence signals within small animals are inherently weak and become even more attenuated after passing through tissue. If the fluorescence noise emitted by the optical fiber is comparable to or stronger than the true biological signal, the real signal will be submerged in noise, leading to detection failure or inaccurate data.

The ultra-low fluorescence fiber solution: This type of fiber utilizes specially doped silica materials (typically characterized by extremely high purity and optimized hydroxyl

(-OH) content) and manufacturing processes, ensuring that almost no autofluorescence is generated under common excitation wavelengths (e.g., 400–900 nm).

6.3 Spectral Unmixing

Modern biomedical research often requires the simultaneous use of multiple probes. However, their spectra overlap, resulting in "crosstalk" interference, similar to mixing multiple pigments, making them difficult to distinguish. If they cannot be effectively unmixed, it is difficult to obtain accurate data.



Mixed spectra → Algorithmic separation → Independent signals

Figure 30 Schematic of Spectral Unmixing

The core of **spectral unmixing** is **spectral deconvolution**. This is a powerful mathematical algorithm that can isolate the contribution of each probe from the mixed spectrum. A robust algorithm must provide accuracy, automation, and traceability to ensure the reliability of quantitative analysis in multicolor experiments.

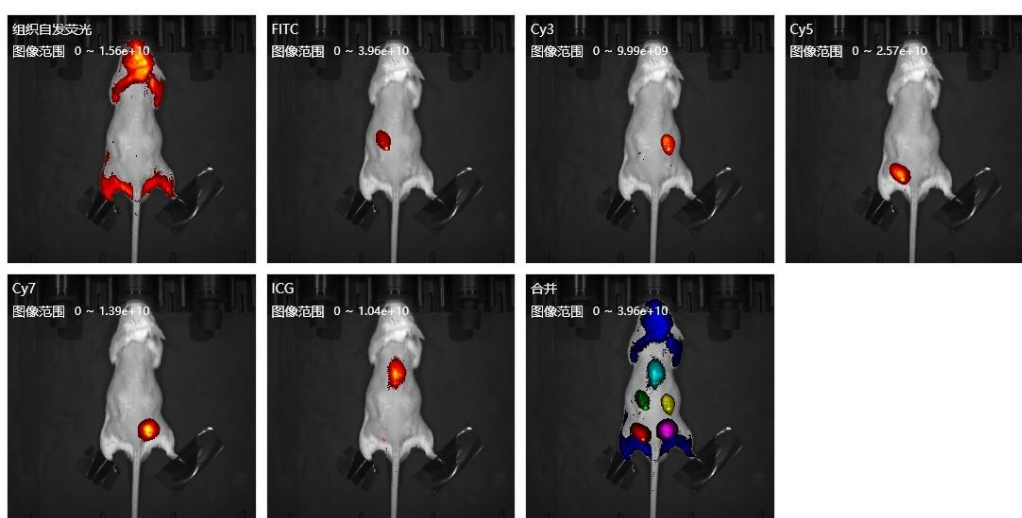


Figure 31 Spectral unmixing results

6.4 Data Correction

In scientific research, the authenticity and reliability of data are fundamental requirements. High-sensitivity imaging systems must be equipped with comprehensive data correction functions to effectively suppress "false positive" signals and control systematic errors, ensuring the reliability and reproducibility of experimental results to meet the high standards required for academic publication.

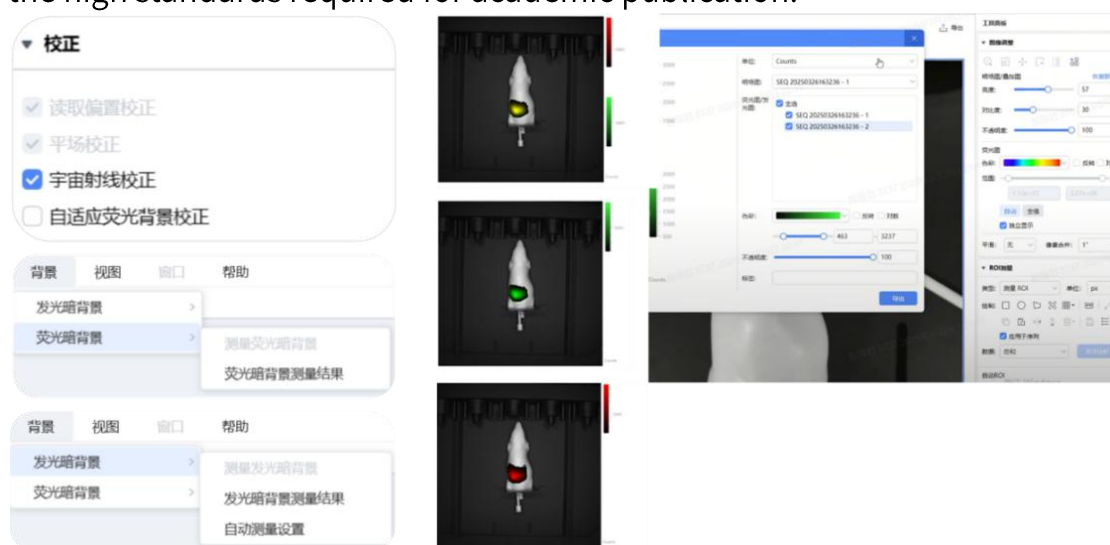


Figure 32 Demonstration of Data Correction Functions

In-depth analysis: Data correction is not optional in in vivo imaging; it is the cornerstone for obtaining reliable, reproducible, and quantitative scientific conclusions. Conclusions drawn from in vivo imaging data that have not undergone rigorous correction are often questionable, or may even be entirely incorrect.

The fundamental challenge in in vivo imaging is that the raw data detected by the system is a mixture of various factors, rather than a direct reflection of the target biological signal. These factors include:

- 1) Non-specific background: Animal autofluorescence (fur), instrument noise, etc.
- 2) Complex photon-tissue interactions: Absorption (e.g., strong absorption of blue-green light by hemoglobin) and scattering, leading to severe signal attenuation and spatial distortion.
- 3) Interference from experimental variables: Probe injection dosage, injection efficiency, individual animal variability (body weight, metabolic rate), etc.
- 4) Inherent instrument bias: Fluctuations in light source intensity, inconsistent camera pixel response, filter efficiency, etc.

Without correcting for these factors, direct comparison of "raw fluorescence values" across different time points, animals, or experimental groups is meaningless.

Data correction is primarily divided into two categories: **instrument hardware correction** and **biological background correction**.

6.4.1 Instrument hardware correction (Ensuring the accuracy of the measurement tool itself)

Flat-field Correction

Slight differences exist in sensitivity across camera sensor pixels; lens vignetting may cause the image center to be brighter than the periphery; and light source illumination may be non-uniform.

Importance: This is the essential first step before performing any quantitative analysis; otherwise, intensities from different regions of the image cannot be directly compared.

Dark-current Correction

Even in the absence of light, camera sensors generate signals due to thermal effects, known as "dark current" or "hot pixels," which appear as fixed-pattern noise in the image. This is particularly severe during long exposures.

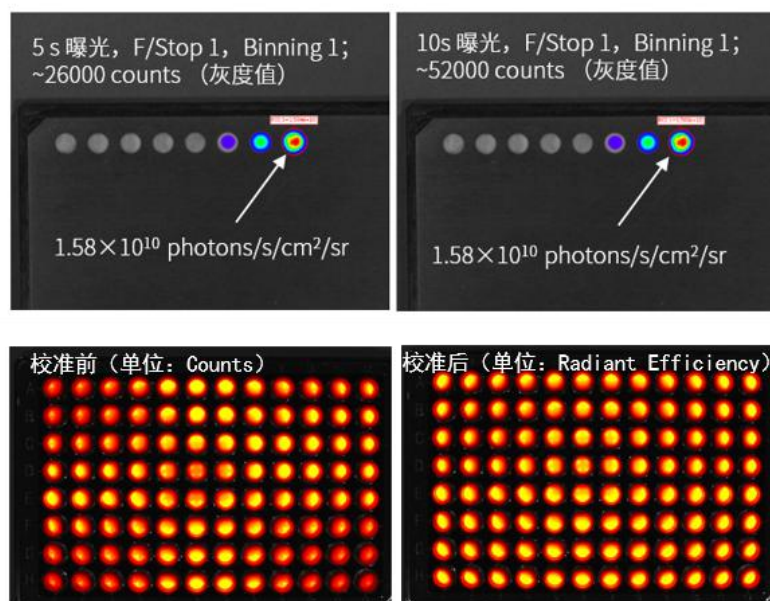
Importance: Eliminating intrinsic camera thermal noise to improve the signal-to-noise ratio (SNR) is critical for detecting weak signals.

6.4.2 Biological Background and Quantitative Correction (Ensuring data reflects biological truth)

Background Fluorescence Subtraction

Problem: Autofluorescence from animal tissues (e.g., skin, fur, internal organs) under excitation light overlaps with the specific signal from the probe.

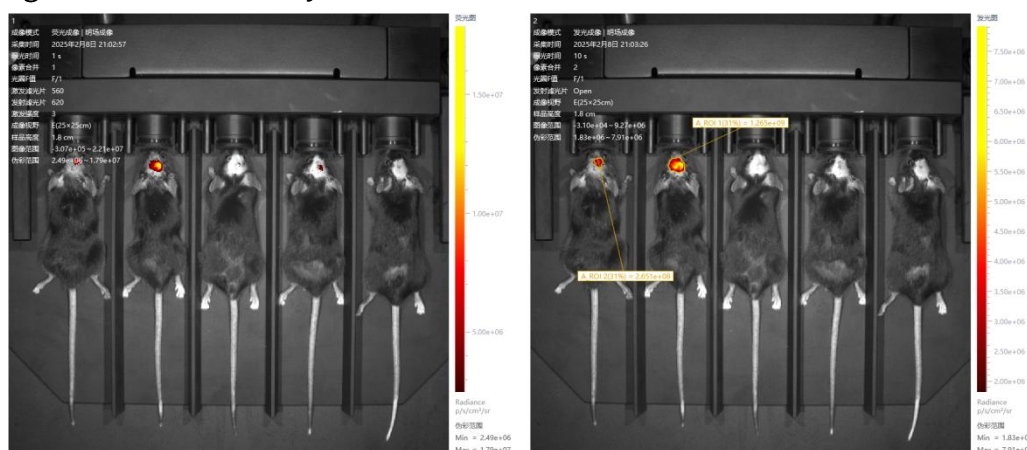
Importance: Obtaining the true net signal of the probe. This is the basis for calculating the target-to-background ratio (TBR).



To ensure reliable and reproducible experimental data, it is recommended to use small animal in vivo imaging equipment that incorporates NIST-traceable light sources and supports excitation light uniformity correction for fluorescence modalities, thereby enabling absolute quantification and precise analysis of bioluminescent/fluorescent signals.

6.5 High-Throughput Design

Modern biomedical research demands higher experimental efficiency. A high-performance in vivo imaging system should possess high-throughput capabilities to meet the demand for rapid, continuous imaging of large batches of samples, significantly enhancing research efficiency.



High-throughput design is reflected in several aspects: a large field of view (FOV) can accommodate multiple animals in a single acquisition, significantly saving time; a multi-animal synchronized anesthesia system ensures efficient and safe operation; and modular upgrades provide greater scalability and a longer service life.

6.6 Integrated Support Services

When choosing a small animal in vivo imaging system, most researchers initially focus on performance parameters (such as sensitivity and resolution) and price. However, a comprehensive service system is often a key factor in determining long-term research success and user experience, providing benefits that are both immense and far-reaching.

1) Professional and objective technical consultation

It is not just about selling products, but about providing solutions. Experienced application specialists recommend the most suitable model and configuration based on the client's specific research field (oncology, neuroscience, infection and immunity, etc.), animal models (mice, rats, zebrafish), experimental requirements (bioluminescence, fluorescence, X-ray), and budget, helping you avoid purchasing equipment with "excessive" or "insufficient" performance.

2) In-depth pre-sales demonstration and verification

Clients can directly observe the imaging results of their own experimental samples, gaining first-hand confidence in the equipment's performance and eliminating the risk of purchasing systems that "look good on paper but are impractical."

3) Efficient and reliable after-sales response

Provide clear localized support channels (phone, email, online customer service), rapid on-site response (e.g., within 24–72 hours), and spare parts supply.

When equipment malfunctions occur, downtime can be minimized. For scientific research, time is everything. Rapid repairs ensure that experimental progress remains uninterrupted, preventing the loss of valuable animal models and samples due to equipment downtime.

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Appendix & Downloadable Resources

Guide to Common Probes, Substrates, and Radionuclides for Small Animal In Vivo Imaging

This guide provides comprehensive information on fluorescent probes, bioluminescent substrates, and radionuclides commonly used in small **animal in vivo imaging (400–900 nm)**, extending to emerging NIR-II (1000–1700 nm) and multimodal probes.

I. Common Fluorescent Probes and Dyes

Probe/Dye	Excitation Peak (nm)	Emission Peak (nm)	Characteristics and Applications
FITC	490	520	The classic green fluorescent dye, commonly used for antibody/protein labeling
Cy5	650	670	Deep tissue imaging with superior tissue penetration
Cy7	750	780	Near-infrared window, low background, high penetration depth
ICG (Indocyanine Green)	780	810	FDA-approved for clinical use with high safety; used for blood flow, tumor imaging, and lymph node mapping
Alexa Fluor Series (488/594/647/750)	Varies	Variable	High brightness, high photostability, commonly used for immunolabeling
IRDye 800CW	774	789	Commonly used for antibody conjugation, widely applied in clinical translational research
DiR	748	780	Lipophilic; labels cell membranes/exosomes for tracking

DiD	644	665	Lipid labeling; commonly used for exosome/cell tracking
Dil	549	565	Neuronal/cell membrane dye, commonly used for tracking
CellTracker series	492/577	517/602	Long-term cell tracking probes, partitionable during cell division
MitoTracker series	490–600	516–650	Mitochondria-specific staining, compatible with live cells
pHrodo series	560/640	585/690	pH-dependent signaling for studying phagocytosis/lysosomal function
ROS probes (DCFH- DA, DHE)	485	530	Detection of intracellular ROS levels
FRET probes	Dual- channel	Dual channel	Study dynamic intermolecular interactions
Activatable probes (MMP, Caspase)	Variable	Variable	Activated by specific enzymes; low background and high SNR

II. Common substrates for bioluminescence

Substrate	Luciferase system	Emission Peak (nm)	Characteristics and Applications
D-Luciferin	FLuc (firefly luciferase)	560–620	Most commonly used, ATP-dependent, stable signal; used for tumor burden/gene expression
AkaLumine-HCl	Akaluc	~650	Red-shifted luminescence, deep-tissue imaging
Coelenterazine (CTZ)	RLuc/GLuc	480–510	ATP-independent, blue light, rapid signal decay, suitable for dynamic superficial imaging
Cypridina Luciferin	VLuc	~460	Blue light, marine origin, often used for in vitro experiments
Benzothiophene luciferin (BtLuc)	FLuc variant	600–650	Red-shifted, improves deep-tissue sensitivity
Furimazine	NanoLuc	~460	Extremely high brightness, hundreds of times brighter than traditional substrates, suitable for weak signal detection
h-Coelenterazine	RLuc8/Antares	560–580	Improved CTZ, higher signal-to-noise ratio
DeepBlueC	RLuc	400–460	Blue light, used in BRET systems
Vargulin	VLuc	460	Marine-derived, ATP-independent, low background

NanoLuc-BRET (NanoLuc + fluorophore)	NanoLuc	460→600–700	Achieves red-shifting via energy transfer to enhance deep-tissue imaging
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III. In Vivo Radionuclide and Cherenkov Luminescence Imaging (CLI)

Radionuclide	Half-life	CLI emission range (nm)	Characteristics and Applications
^{18}F (e.g., FDG)	110 min	500–700	Most commonly used PET radionuclide; enables PET/CLI dual-modality imaging
^{64}Cu	12.7 h	500–750	Moderate half-life, suitable for antibody labeling
^{131}I	8 d	500–700	Therapeutic radionuclide; beta particles generate CLI signals
^{90}Y	2.7 d	500–750	High-energy beta emitter, commonly used in tumor therapeutic probes
^{68}Ga	68 min	500–700	Short half-life, suitable for small peptide probes
^{177}Lu	6.6 d	500–750	Therapeutic radionuclide; can also be monitored via CLI
^{211}At	7.2 h	500–700	Alpha emitter with therapeutic and imaging potential
$^{99\text{m}}\text{Tc}$	6 h	500–700	Common SPECT radionuclide, CLI applications have been explored
^{125}I	60 d	500–650	Long-term labeling; low-energy gamma emitter suitable for CLI

IV. Multimodal probes and special functional probes

Probe Category	Representative Probe	Excitation/Emission Peak (nm)	Characteristics and Applications
Photoacoustic /Fluorescence Dual-mode Probe	ICG-nanoparticles, IR780	780/810	Fluorescence + photoacoustic; combines sensitivity and resolution; suitable for tumor vascular imaging
Magnetic Resonance/Fluorescence Dual-mode Probe	SPIO-ICG	780/810	Combines high MRI resolution with high NIRF sensitivity
Radionuclide/Fluorescence Dual-mode Probe	⁶⁴ Cu-ICG	PET (511 keV) / 810 nm	High PET sensitivity + real-time fluorescence localization for pre-operative/intra-operative integration
pH/Oxygen-sensitive Probe	Pt(II)-porphyrins, Ru(II) complexes	400–450/600–700	Sensitive to hypoxic or acidic environments, reflecting tumor or ischemic status
Bioresponsive Probe	Caspase-3/7 Activatable Probe	~500/600	Emits light upon specific enzyme activation; low background; monitors apoptosis

V. Emerging and Special Application Probes

Probe/Dye	Excitation Peak (nm)	Emission Peak (nm)	Characteristics and Applications
Quantum Dots (QDs)	400–600	500–800 (tunable)	Ultra-high brightness, excellent photostability, multicolor imaging; Disadvantages: potential toxicity
Near-infrared-II (NIR-II) Probes	808/980	1000–1700	Deep penetration, high SNR, suitable for deep brain/liver imaging
Genetically Encoded Fluorescent Proteins (iRFP720, mCherry)	690 / 587	720 / 610	Stable expression via genetic engineering, long-term tracking, no injection required
Integrated Photothermal/Fluorescence Probes (Au nanorods, CuS nanoparticles)	750–800	800–850	Combines photothermal therapy and fluorescence imaging
Integrated Photodynamic/Fluorescence Probes (high-dose ICG, porphyrins)	630–780	650–810	Provides both photodynamic therapy and fluorescence guidance
ATP probe (ATeam, FRET-based)	CFP/YFP pair	480/530	Real-time monitoring of ATP concentration changes based on FRET
Membrane potential probes (DiBAC4(3), RH414)	480–530	520–600	Reversible signal changes with membrane potential; used for neural activity imaging

Ion probes (Fluo-4, SBFI)	494 / 340	516 / 505	Calcium/sodium-specific probes, commonly used in neuroscience
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VI. Precautions for Probe Preparation and Application

Category	Key Precautions and Recommendations
Probe Selection	Wavelength matching (filters/light source), efficient conjugation, biocompatibility
Route of administration	IV is most common; IP absorption is slower; SC for local imaging; PO mainly for the GI tract
Imaging process	Stable anesthesia (isoflurane); background subtraction before injection; optimal exposure to avoid saturation
Data analysis	ROI quantification; multimodal registration; time-point dynamic analysis

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RWD aims to provide customers with end-to-end solutions from live cells to in vivo studies. We currently offer a complete product evidence chain starting from single-cell isolation and culminating in mechanistic validation.

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