Bioimaging with Upconversion Nanoparticles

Evan M. Mettenbrink, Wen Yang, and Stefan Wilhelm*

Bioimaging enables the spatiotemporal visualization of biological processes at various scales empowered by a range of different imaging modalities and contrast agents. Upconversion nanoparticles (UCNPs) represent a distinct type of such contrast agents with the potential to transform bioimaging due to their unique optical properties and functional design flexibilities. This review explores and discusses the opportunities, challenges, and limitations that UCNPs exhibit as bioimaging probes and highlights applications with spatial dimensions ranging from the single nanoparticle level to cellular, tissue, and whole animal imaging. Recent advancements in bioimaging applications enabled by UCNPs, including super-resolution techniques and multimodal imaging methods are summarized, and a perspective on the future potential of UCNP-based technologies in bioimaging research and clinical translation is provided. This review may provide a valuable resource for researchers interested in exploring and applying UCNP-based bioimaging technologies.

1. Introduction

Bioimaging enables the spatiotemporal visualization of biological processes at scales ranging from the molecular level to whole organisms using various techniques and modalities. These modalities include light, ultrasound, magnetic resonance, X-rays, and other types of radiation to capture processes within complex biological systems.^[1–3] To facilitate the visualization of these processes and to enhance the imaging contrast, nanoparticles are often used.^[4] Advancements in bioimaging facilitate the use of a unique type of nanoparticles, called upconversion

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nanoparticles (UCNPs), that show promise due to their ability to be applied with many imaging modalities, including optical imaging.^[5]

UCNPs are often made from crystalline inorganic host materials doped with lanthanide ions and exhibit overall dimensions in the nanoscale size range (1-100 nm).^[6] These lanthanide ions in combination with the host material enable the unique optical properties of UCNPs, resulting in anti-Stokes luminescence.^[7] The anti-Stokes (or upconversion) luminescence is generated by the sequential absorption of multiple low-energy (or longer wavelength) photons followed by the emission of a photon with relatively higher energy (or shorter wavelength).^[8–10] The photon upconversion phenomenon has been used in a wide array of applications, including solar

cell enhancement,^[11] biological and chemical sensing,^[12–16] photodynamic therapy,^[17] diagnostic devices,^[18,19] photoactivated gene editing,^[20] and even to give mice infrared vision.^[21]

While sometimes criticized for their relatively low overall quantum yields when compared to fluorescent probes and quantum dots. UCNPs have vast potential in bioimaging due to a unique combination of properties and capabilities not found in other imaging probes, including: 1) a relatively high absorption cross-section of lanthanides, the essential dopant ions of UCNPs, in the near-infrared (NIR) range paired with multiphoton upconversion capabilities to enable excitation and emission in the 700-1,000 nm range where biological tissue shows relatively low attenuation, known as the NIR-I optical window.^[22] 2) An ability to absorb two, three, or more photons and emit multiple wavelengths across the ultraviolet, visible, and NIR spectra enables applications in super-resolution imaging, multiplexed bioimaging, and tuning of emission spectra.^[23,24] 3) Energy transitions with extended time frames and nanoparticle architecture engineering allow for wide spans of emission lifetimes ranging from micro- to milliseconds.^[25] 4) A non-blinking, photostable, and reliable luminescence signal enabling stable imaging for accurate comparative, or long-term observational studies.^[26] 5) Luminescence origins within the nanoparticle, allowing for an ever-expanding library of surface modifications to enable targeting, sensing, energy transfer, prolonged circulation/reduced toxicity upon exposure to biological systems, or light-activated nanodevices with minimal effects on luminescence.^[27-30] 6) Ease of compositional modification and tunability to enable X-ray, magnetic resonance, photoacoustic, or single-photon emission computed tomography-based multimodal imaging for added potential in clinical applications.^[31]



While fluorescent probes may have NIR capabilities and are being developed for multi-photon applications, these probes often exhibit limited photostability, have a substantially lower range of possible emission lifetimes,^[32] have limited upconversion capabilities, and typically lack the multimodal potential found in UCNPs. Quantum dots and carbon dot nanoparticles exhibit relatively higher photostability, but only recent research has enabled their use in NIR–NIR imaging.^[33] In addition, these nanoparticles are often prone to photoblinking^[34] and have limited capabilities regarding photon upconversion than UCNPs.^[35]

To investigate the intersection of bioimaging and UCNPs, this review seeks to introduce the readers to the photophysical mechanisms, advantages, and limitations, as well as applications of UCNPs. We highlight recent advancements in the field before focusing on how UCNPs address the immediate needs for bioimaging probes at the individual nanoparticle level, including upconversion luminescence (UCL) tunability, photostability, quantum yield, and super-resolution microscopy; the cellular level, including UCNP cytotoxicity, surface modification, multiplexing, and enacting cellular processes; and the whole animal level, including UCNP biocompatibility and biodistribution, imaging depth, invivo targeting, and multimodal imaging. Additionally, this review provides a perspective on the current state of UCNP bioimaging and identifies opportunities and challenges in advancing this technology. Finally, we hope this review will serve as a valuable resource for researchers who wish to

explore UCNPs as a potential technology for enhancing their bioimaging experiments.

2. Basic Concepts of Upconversion Nanoparticles

2.1. The Photophysical Mechanisms of Photon Upconversion

To achieve photon upconversion, UCNPs often employ two classes of lanthanide ions, called the sensitizer and activator ions, added to the crystalline UCNP host material during synthesis. The sensitizer ion (often ytterbium) has an absorbance peak in the NIR range, typically \approx 980 nm, and can transfer energy in its excited state to activator ions (often erbium, thulium, or holmium, **Figure 1**).^[2] To enable this energy transfer, the UCNP design requires the selection of lanthanide ions with matching or closely matching excited energy levels, enabling electron transfer between them. These energy levels further dictate the wavelength of the emitted (upconverted) photon.^[36]

The emission wavelength will vary depending on which activator energy state the electrons occupy. For example, the ${}^{4}F_{9/2} \rightarrow {}^{4}I_{15/2}$ and ${}^{4}I_{13/2} \rightarrow {}^{4}I_{15/2}$ electron energy transitions in erbium result in a ≈ 660 and $\approx 1,532$ nm wavelength photon, respectively, and the ${}^{1}G_{4} \rightarrow {}^{3}H_{6}$ transition in thulium results in the emission of a ≈ 478 nm wavelength photon (Figure 1). ${}^{[37-40]}$ Since each of these energy transitions involves the absorption



Figure 1. Simplified schematic representation of photophysical properties of upconversion nanoparticles (UCNPs). Colloidal dispersions of: a) β -NaYF4(Yb³⁺/Er³⁺) UCNPs and b) β -NaYF4(Yb³⁺/Tm³⁺) UCNPs in cyclohexane with corresponding upconversion luminescence (UCL) spectra upon 980 nm continuous wave (CW) laser excitation (10 W cm⁻²). Simplified energy-level diagrams and energy transfer upconversion mechanisms for: c) Yb³⁺/Er³⁺-doped and d) Yb³⁺/Tm³⁺-doped sensitizer/activator ion systems. The excitation light (980 nm) is absorbed by Yb³⁺ sensitizer ions and sequentially transferred to Er³⁺/Tm³⁺ activator ions. Arrows indicate radiative, nonradiative energy transfer, and multiphonon relaxation processes.^[5] Adapted with permission.^[5]. Copyright 2017, American Chemical Society.



and transfer of different numbers of photons, a single UCNP will typically exhibit multiple prominent upconversion emission peaks upon excitation. Notably, anti-Stokes shifts between excitation and emission wavelengths as high as \approx 1,200 nm are possible.^[36,39,41,42] If multi-wavelength emissions are unwanted in a specific application, the photophysical upconversion processes can be engineered to isolate specific emission wavelengths by targeting electron energy transitions through modulation of the excitation laser.^[43]

Upconversion nanoparticles primarily employ the following three photophysical processes to generate upconverted photons: 1) excited state absorption (ESA), 2) energy transfer upconversion (ETU), or 3) photon avalanche (PA).^[23,37,44–46] Notably, other anti-Stokes emitting processes exist, including multi-photon absorption and second harmonic generation.^[47,48] However, a key difference in these processes is that second harmonic generation and multi-photon absorption require the rapid, near-simultaneous absorption of photons as the electrons do not occupy a true intermediate energy state. In comparison to the virtual intermediate of these processes, UCNPs employ true

intermediate states.^[36] A primary advantage of the real intermediate states is that it enables upconversion luminescence (UCL) without using more costly femtosecond lasers. A summary schematic of these photophysical processes is shown in **Figure 2**.^[49]

It is worth noting that a common form for the nomenclature of UCNPs is to start with the core matrix, followed by the activator and sensitizer ions along with their doping ratios, and then an "@" sign denoting each additional shell around the core matrix. For example, a NaYF₄:20% Yb³⁺, 2% Er³⁺@ NaYF₄ UCNP would have a sodium yttrium fluoride core matrix with a ytterbium sensitizer and an erbium activator surrounded by a shell made of sodium yttrium fluoride. The UCNP's core matrix would contain the following mol percentages: 78% yttrium, 20% ytterbium, and 2% erbium.

2.2. Imaging in the NIR Range

An important feature of UCNPs is that they enable imaging in the NIR range with typical excitation wavelengths of \approx 808 or \approx 980 nm. In some circumstances, even longer wavelengths have





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Table 1. Select examples of bioimaging applications with upconversion nanoparticles.

UCNP Composition ^{a)}	UCNP Size [nm]	Demonstrated Application	Imaging Depth	Excitation/ Emission [nm]	Ref.
Image Enhancement					
NaYF₄@NaYbF₄@NaYF₄:10–99% Yb/1% Tm@NaYF₄@PAA	6–49	In vivo lifetime multiplexing of UCNPs	6 mm	980	[53]
LiF4:99% Er/1% Tm@LiYF4	8	Imaging onion microstructures	Imaged in onion cells	980/Visible	[54]
NaF4:40% Yb/60% Er@NaYGdF4	12	Low excitation intensity upconversion imaging	Imaged in mammary pads 3-4 mm deep	980/530–550	[55]
NaF4:98% Yb/2% Er@CaF2	14	Emission intensity optimization for naked eye-visible UCNPs	Visible in mouse forearm	980/Visible	[56]
NaYF4:20% Gd/0.5% Tm@NaGdF4	15	Energy-looping nanoparticles for imaging through brain tissue phantoms	1 mm	1064/800	[57]
NaGdF ₄ :20% Yb/2% $Er@CaF_2$	16	Novel cation exchange UCNP synthesis method as well as shell UCL enhancement	Imaged near mouse hip	980/<680	[58]
NaLuF₄:20% Yb/1% Tm	19	Increase in contrast and reduction in tissue overheating using time-gating imaging approach	Imaged near mouse side	980	[59]
NaYF4:20% Yb/2% Tm	20	Early paper showing in vivo imaging of UCNPs	Imaged in mouse abdomen	975/800	[60]
NaYF₄:20% Yb/2% Er@polyglutamic dendron	23	Mapping mouse vasculature as well as pH sensing with low power excitation	0.2 mm	980, 808	[61]
NaYF4:20% Yb/8% Tm@PEI	25	Time-gating approach for the reduction in background fluorescence	Imaged in excised spleen	975/455	[62]
NaYF4:Mn2+/18% Yb/2% Er@PEG	25 (no PEG)	In vivo imaging of mice following injection of particles	15 mm	980/<800	[40]
NaYF ₄ :20% Yb/0.5% Tm	≈25	Super-resolution technique for resolving lines through liver tissue with increased speed of imaging	0.051 mm	976/800	[51]
NaF4:66% Yb/30% Gd/2% Ce/2% Er@NaYF4:10% Yb@Ag2Se QDs	26	Quantum dot-sensitized UCNPs for imaging of traumatic brain injury	11 mm	980/>1300	[63]
NaF4:99.5% Er/0.5% Tm@NaYF4@SiO2	28	Multi-excitable UCNPs with enhanced upconversion	Imaged near mouse hip	1532, 980, 808/Short pass	[64]
NaYF4:80% Yb/6% Er@NaYF4	28	Imaging of nanoparticles in vivo using minimal excitation power	4 mm	980/647–673	[65]
NaYF₄:20%Yb/2% Er@Dendrimer	30	Deep mapping of mouse brain vasculature at low excitation power	1 mm	980/<890	[66]
NaYF ₄ :40% Yb/4% Tm	41	Super-resolution techniques for imaging individual nanoparticles through liver tissue	0.093 mm	980/<842	[67]
NaGdF₄:20% Yb/2% Er @ BSA∙DTPAGd	43	Strategies for gadolinium integration for MRI in mice	MRI only	N/A	[68]
ZrOCl ₂ :3.14% DCDPA/3.14% Pd- TCPP	55	Metal-organic alternative framework for low power density (0.005 W cm ⁻²) of lymph nodes	Imaged subcutaneous lymph nodes	532/425–475	[69]
NaYF₄:20% Yb/5% Gd/2% Er@NaYF₄@Ag₂Se QDs	62	Enhancement of PDT and luminescence activity through quantum dots	Imaged in mouse back	800	[70]
NaYF4:30% Yb/0.5% Tm@NaYF4@SiO2@OTMS@F127	84	Brain vessel mapping and in vivo UCL imaging	Imaged in mouse chest	980/750–900	[71]

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Table 1. Continued.

UCNP Composition ^{a)}	UCNP Size [nm]	Demonstrated Application	Imaging Depth	Excitation/ Emission [nm]	Ref.
Novel Architectures and Surface Coating	gs				
$K_{3}ZrF_{7}:20\%$ Yb/2% Er	27	Monitoring biodegradable nanoparticles in vivo	\approx 7 mm	980/Visible	[72]
NaGdF4:70% Yb/1% Tm@NaGdF4@Poly-d-lysine/DNA	38	Specific miRNA-enhanced luminescence for detection in mice	Imaged near mouse hip	980	[52]
NaGdF4:18% Yb/2% Tm@NaGdF4@Cancer Cell Membrane	48	Multimodal targeting and differentiation between triple- negative and MCF7 breast cancer cells	Imaged near mouse rear	980/790	[73]
ZrOCl2:3.14% DCDPA/3.14% Pd-TCPP	55	Metal–organic alternative framework for low power density (0.005 W cm ⁻²) of lymph nodes	Imaged subcutaneous lymph nodes	532/425-475	[69]
NaYF₄:18% Yb/0.6% Tm@NaYF₄@PMAO/PEG	75	Imaging UCNP accumulation in tumors using an amphiphilic polymer coating	Imaged in mouse lungs	975/Visible	[74]
NaYF4:18%Yb/0.6% Tm@NaYF4@PMAO	75	Simultaneous bioimaging and local light-activated hyperthermia	Imaged near mouse shoulder	980/800	[75]
NaYF₄:18% Yb/2% Er@SiO₂@AuNP@DNA hairpin	≈75	DNA-functionalized UCNPs for biocompatibility, deep tissue imaging, and guided drug release	Imaged in mouse abdomen	980/750–830	[76]
NaYF ₄ :30% Yb/1% Er@Cancer cell membrane	80	Cancer cell-coated UCNPs for tumor targeting and imaging	Imaged throughout mice	980/535	[77]
NaYF ₄ :30% Yb/1% Er@RBC proteins	90	Erythrocyte membrane-coated UCNPs for increased biocompatibility	Imaged near mouse hip	980/535	[29]
NaYF₄:20% Yb/2% Tm@NaYF₄@PEI@Colomnic acid	90	Colomnic acid prolongs circulation and enables blood vessel and inflammation imaging	Imaged in mouse blood vessels	975	[78]
NaYF4:20% Yb/0.5% Tm@NaGdF4: Yb@g-C3N4/Au25/PEG	110	Graphitic-phase carbon and gold nanocluster-mediated ROS generation for PDT & trimodal MRI/CT	Imaged in mouse shoulder (Supplemental)	980	[79]
NaYF ₄ :20% Yb/2% Er@PEG/ ¹²⁵ I	120	SPECT/CT tracking of radiolabeled nanoparticles	Whole-body SPECT	N/A	[80]
NaYF₄:20% Yb/2% Tm@NaYF₄@PMAO/DARPin	213	DARPin-mediated targeting of HER2 positive cells in xenograft tumor	Imaged in mouse leg	980/485-831	[81]
NaYF₄:18% Yb/2% Tm@NaYF₄:21.4% Yb/21.4% Nd@CNQds in nanobubbles	428	PDT through light-mediated carbon nitride quantum dot ROS generation and ultrasound-mediated release	Imaged near mouse hip	808/830+	[82]
In Vivo Targeting/Sensing					
NaGdF4:28% Yb/2% Er/10% Ce@PEG/cMBP	13	Targeting of overexpressed squamous cell cancer protein for multimodal diagnosis	N/A	808	[83]
NaF4:99.5% Er/0.5% Tm@NaYF4@NaGdF4:15% Tb@Folic acid	17	X-ray activated PDT and tumor targeting	Imaged subcutaneous tumor	980	[84]
NaGdF₄:35% Yb/0.5% Tm@NaGdF₄@PEG-FA/PC70	20	Fluorescent, UCL, and MRI imaging of tumor-targeted nanoparticles for PDT	Imaged near mouse hip	980	[85]
NaYF₄:20% Yb/2% Er@polyglutamic dendron	23	Mapping mouse vasculature as well as pH sensing with low power excitation	0.2 mm	980, 808	[61]F
NaF4:15% Yb/85% Er@NaGdF4:20% Yb@SiO2	23	Tumor targeting and comparison of peptide performance for colorectal cancer identification	Imaged in mouse colon	980/<675	[86]



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Table 1. Continued.

UCNP Composition ^{a)}	UCNP Size [nm]	Demonstrated Application	Imaging Depth	Excitation/ Emission [nm]	Ref.
NaF4:98% Er/2% Ho@NaYF4	24	Patch for in vivo sensing of $\rm H_2O_2$ and inflammation	Imaged UCNPs on dermal patch	1530/1180 & 980	[87]
NaGdF4:70% Yb/1% Tm@NaGdF4@Poly-d-lysine/DNA	38	Specific miRNA-enhanced luminescence for detection in mice	Imaged near mouse hip	980	[52]
NaYF4:20% Yb/2% Er@NaYF4:10% Yb/40% Nd@NaYF₄@PEG/ANG2	42	Passing through the blood-brain barrier and light-mediated endolysosomal escape for metronomic chemotherapy	Imaged in mouse brain	808 PDT. 980/<950	[88]
NaGdF4:18% Yb/2% Tm@NaGdF4@Cancer Cell Membrane	48	Multimodal targeting and differentiation between triple- negative and MCF7 breast cancer cells	Imaged near mouse rear	980/790	[73]
NaYbF4:2% Er@NaYF4:10% Yb@NaYF4:40% Nd, 10% Yb@PAA- Rh1000	57	Hypochlorous acid detection for bioimaging of arthritis in mice.	Imaged in mouse legs	980 & 540	[89]
Na(Y/ ⁹⁰ Y)F₄:20% Yb/0.6% Tm@PMAO@ DARPin-PE40	75	DARPin for targeted, 2200 times synergistic therapeutic increase with PE40/radioactive yttrium-mediated therapeutics against HER2+ breast cancer cells.	Imaged in mouse thigh	980/485–831	[90]
NaYF₄:18% Yb/2% Er/Mn@IR-780/ mTHPC/ANG2	80	Targeted PDT of intravenously injected UCNPs for glioblastoma treatment	Imaged in brain and excised organs	675/730-760	[91]
NaYF4:30% Yb/1% Er@Cancer cell membrane	80	Cancer cell-coated UCNPs for tumor targeting and imaging	Imaged throughout mice	980/535	[77]
NaYF4:18% Yb/2% Er@NaGdF4@PEG/CD326mAb	85	MRI/UCL to monitor the antibody- dependent increase in UCNP uptake for pancreatic cancer detection	Imaged near mouse shoulder	980/650	[92]
NaGdF4:18% Yb/2% Tm/2% Ca@NaLuF4@PEG/anti-HER mAb	115	SPECT/CT/UCL imaging for metastatic lymph node detection, prolonged circulation, and tumor targeting	7.7 mm	980	[93]
NaYF4:5% Nd@BDM/PtTPBP	165	In vivo sensitive temperature sensing	5 mm	808, 638/485–575, 980-1300	[13]
NaYF4:20% Yb/2% Tm@NaYF4@PMAO/DARPin	213	DARPin-mediated targeting of HER2 positive cells in xenograft tumor	Imaged in mouse leg	980/485-831	[81]
PdTPBP/(perylene or BPEA) @SiO ₂ @peptide	216	Multiple UCNP injections for single-excitation identification of two tumor types <i>in vivo</i>	Imaged near mice hips	635/515 or 475	[94]
Photodynamic Therapy					
NaF4:99.5% Er/0.5% Tm@NaYF4@NaGdF4:15% Tb@Folic acid	17	X-Ray activated PDT and tumor targeting	Imaged subcutaneous tumor	980	[84]
NaGdF₄:35% Yb/0.5% Tm@NaGdF₄@PEG-FA/PC70	20	Fluorescent, UCL, & MRI imaging of tumor-targeted nanoparticles for PDT	Imaged near mouse hip	980/Not Listed	[85]
NaGdF4:Yb/Er@Ce6/DNA	20	MRI/CT/UCL/PA Imaging-guided ce6-mediated PDT	Imaged near mouse hip	980	[95]
NaErF ₄ @NaYF ₄ @NaYbF ₄ :0.5% Tm@NaYF ₄ @TiO ₂	40	Imaging-guided PDT using titanium dioxide-mediated ROS generation	Imaged in mouse chest	980 PDT, 808/Not listed	[17]
NaYF4:20% Yb/2% Er@NaYF4: Yb@PDA/ICG	40	PDT of injected nanoparticles	N/A	808	[96]
NaYF4:20% Yb/2% Er@NaYF4:10% Yb/40% Nd@NaYF4@PEG/ANG2	42	Passing through the blood-brain barrier and light-mediated endolysosomal escape for metronomic chemotherapy	Imaged in mouse brain	808 PDT. 980/<950	[88]

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Table 1. Continued.

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UCNP Composition ^{a)}	UCNP Size [nm]	Demonstrated Application	Imaging Depth	Excitation/ Emission [nm]	Ref.
NaGdF4:20% Yb/2% Er@NaGdF4:30% Nd/10% Yb@IR- 808@Ce6/MC540 SiO2	46	Dye sensitization for increased PDT with minimal heating effects & trimodal UCL/CT/MRI	MRI/CT near mouse shoulder	808	[97]
NaGdF₄:18% Yb/2% Er/2% Co/3% Mn@SiO₂/FITC/CuS/ZnPc/DOX	48	CT/MRI multimodal imaging for PDT application	N/A	980	[98]
NaYF4:20% Yb/5% Gd/2% Er@NaYF4@Ag2Se QDs	62	Enhancement of PDT and luminescence activity through quantum dots	Imaged in mouse back	800	[70]
NaYF4:18% Yb/0.6% Tm@NaYF4@PMAO	75	Simultaneous bioimaging and local light-activated hyperthermia	Imaged near mouse shoulder	980/800	[75]
NaYF₄:18% Yb/2% Er@SiO₂@AuNP@DNA hairpin	≈75	DNA-functionalized UCNPs for biocompatibility, deep tissue imaging, and guided drug release	Imaged in mouse abdomen	980/750-830	[76]
NaYF₄:18% Yb/2% Er/Mn@IR-780/ mTHPC/angiopep-2	80	Targeted PDT of intravenously injected UCNPs for glioblastoma treatment	Imaged in brain and excised organs	675/730–760	[91]
NaYF₄:20% Yb/0.5% Tm@NaGdF₄: Yb@g-C₃N₄/Au ₂₅ /PEG	110	Graphitic-phase carbon and gold nanocluster-mediated ROS generation for PDT & trimodal MRI/CT	Imaged in mouse shoulder (Supplemental)	980	[79]
NaYF₄:18% Yb/2% Tm@NaYF₄:21.4% Yb/21.4% Nd@CNQds in nanobubbles	428	PDT through light-mediated carbon nitride quantum dot ROS generation and ultrasound-mediated release	Imaged near mouse hip	808/830+	[82]
Multimodal Imaging					
NaGdF4:28% Yb/2% Er/10% Ce@PEG/cMBP	13	Targeting of overexpressed squamous cell cancer protein for multimodal diagnosis	N/A	808	[83]
NaGdF₄:35% Yb/0.5% Tm@NaGdF₄@PEG-FA/PC70	20	Fluorescent, UCL, & MRI imaging of tumor-targeted nanoparticles for PDT	Imaged near mouse hip	980/Not Listed	[85]
NaGdF4:Yb/Er@Ce6/DNA	20	MRI/CT/UCL/PA Imaging-guided ce6-mediated PDT	Imaged near mouse hip	980	[95]
NaGdF₄:18% Yb/2% Er@PEG	21	Bimodal X-Ray & UCL imaging to monitor consumed UCNPs escape from the digestive tract	Imaged in surgically removed organs	980/528–552	[99]
NaYF₄:20% Yb/2% Er/1% Tm@GdCl₃ @Aminocaproic Acid@ ¹⁸ F	30	Multimodal UCL/MRI/PET imaging	Imaged in mouse chest organs	980/800	[100]
NaGdF₄:20% Yb/2% Er@ BSA·DTPAGd	43	Strategies for gadolinium integration for MRI in mice	MRI only	N/A	[68]
NaGdF4:20% Yb/2% Er@NaGdF4:30% Nd/10% Yb@IR- 808@Ce6/MC540 SiO2	46	Dye sensitization for increased PDT with minimal heating effects & trimodal UCL/CT/MRI	MRI/CT near mouse shoulder	808	[97]
NaGdF4:18% Yb/2% Tm@NaGdF4@Cancer Cell Membrane	48	Multimodal targeting and differentiation between triple-negative and MCF7 breast cancer cells	Imaged near mouse rear	980/790	[73]
NaGdF₄:18% Yb/2% Er/2% Co/3% Mn@SiO₂/FITC/CuS/ZnPc/DOX	48	CT/MRI multimodal imaging for PDT application	N/A	980	[98]
NaYF4:18% Yb/2% Er@NaYF4:10% Yb@NaF4:90% Nd/10% Yb@NaYF4@NaGdF4@HDA-G2	54	Deep multimodal imaging through PAI, MRI, and UCL	Imaged in mouse rear, 25 mm experimental	800/<700	[101]
NaYF4:20% Yb/2% Er@NaYF4:10% Yb/30% Nd@SiO2/ICG	62	Photoacoustic enhancement using UCNP/indocyanine green for mouse brain and depth imaging	Imaged mouse brain (PAI)	800	[102]

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Table 1. Continued.

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UCNP Composition ^{a)}	UCNP Size [nm]	Demonstrated Application	Imaging Depth	Excitation/ Emission [nm]	Ref.
NaYF4:18% Yb/2% Er@NaGdF4@PEG/CD326mAb	85	MRI/UCL to monitor the antibody- dependent increase in UCNP uptake for pancreatic cancer detection	Imaged near mouse shoulder	980/650	[92]
NaYF4:20% Yb/0.5% Tm@NaGdF4: Yb@g-C3N4/Au25/PEG	110	Graphitic-phase carbon and gold nanocluster-mediated ROS generation for PDT & trimodal MRI/ CT	Imaged in mouse shoulder (Supplemental)	980	[79]
BiF3:20% Yb/2% Er	≈110	Bimodal X-Ray & UCL imaging	Imaged following intraperitoneal injection	980/<700	[103]
NaGdF4:18% Yb/2% Tm/2% Ca@NaLuF4@PEG/anti-HER mAb	115	SPECT/CT/UCL imaging for metastatic lymph node detection, prolonged circulation, and tumor targeting	7.7 mm	980	[93]
NaYF4:20% Yb/2% Er@PEG/ ¹²⁵ I	120	SPECT/CT tracking of radiolabeled nanoparticles	Whole-body SPECT	N/A	[80]

^{a)}Non-exhaustive list of uses of UCNPs in tissue/whole-body imaging grouped by application in image enhancement, surface coating, in vivo targeting capabilities, photodynamic therapy, and multimodal imaging and organized by particle size. Publications applicable to multiple categories are listed multiple times. If articles used multiple particle architectures, the architecture and size used in the tissue/in vivo bioimaging application are listed in the table. In the excitation/emission wavelength column, if an imaging application uses multiple excitations or emissions, the wavelengths are separated by commas, and a slash separates excitation from emission wavelengths.

been used (**Table 1**). The primary advantages of NIR imaging are deeper tissue penetration of the light and reduced phototoxicity of the tissue upon exposure to incident light.^[51] Additionally, tissue components, including erythrocytes and collagen, display autofluorescence when exposed to visible light excitation sources. This autofluorescence increases the signal background and decreases the signal-to-noise ratio in tissue imaging experiments. Photon upconversion imaging with NIR excitation circumvents these autofluorescence issues.^[52]

The enhanced light penetration by NIR light was modeled by Ash et al. and is summarized in **Figure 3**A.^[104] The figure depicts the corresponding tissue depth at which incident light is reduced to 1% of its original intensity in a dermal model. The observed increase in light penetration is due to the chromophores in dermal tissues having lower extinction coefficients at longer wavelengths.^[104] The light attenuation in tissue is driven primarily by two factors: 1) absorbance, i.e., light absorption by tissue components, including molecules and ions, and 2) scattering, i.e., incident light being redirected when traveling through tissue often due to changes in refractive index in tissue components (Figure 3B).^[105–108]

As shown in Figure 3C, NIR-I (700–1,000 nm) light exhibits reduced absorbance (and extinction, i.e., the combination of absorption and scattering) in tissue, which enables deeper light penetration. In addition, these longer wavelengths cause reduced tissue phototoxicity than shorter wavelengths, enabling the use of increased laser power without increasing the energy being transmitted to the tissue, which may otherwise result in tissue hyper-thermia and cell death.^[111,112] This deeper light penetration of tissue and reduction of phototoxic effects on tissue provide NIR UCNPs with enhanced utility in full-body in vivo bioimaging.

Within the NIR-I optical window (700-1,000 nm), the performance of excitation lasers is not all equal. For example, early UCNP research focused more heavily on the 980 nm laser excitation due to ytterbium, the most common UCNP sensitizer, having a relatively strong absorbance peak at \approx 980 nm.^[113] However, as shown in Figure 3C, water exhibits a local absorption maximum in the 980 nm wavelength range, causing a reduction in imaging depth when using \approx 980 nm lasers in comparison to 808 nm lasers, with one study finding 808 nm lasers penetrate 50% deeper than a 980 nm excitation laser in tissue.^[109] Additionally, 808 nm lasers, due to the decrease in water's light absorbance, lead to a reduction in photothermal effects in tissue and associated phototoxicity.^[40,88] Potentially even further supporting the benefits of using 808 nm excitation lasers, Nd³⁺, commonly used instead of or in tandem with Yb³⁺ to sensitize UCNPs to 808 nm excitation, exhibits a relatively stronger absorbance at 808 nm than Yb3+ at 980 nm, leading to an increase in UCL in UCNPs doped with both ions.^[101]

2.3. Tunability

An additional characteristic of UCNPs is their tunability of the corresponding emission lifetimes. This emission lifetime tunability can be achieved by selecting specific ions or combinations of ions. For example, UCNPs with a commonly used activating ion, Tb^{3+} , exhibit an emission lifetime lasting 3.64 ms, whereas Er^{3+} -doped UCNPs exhibit a notably shorter emission lifetime of 0.13 ms.^[114] This phenomenon is exploited in time-gated imaging, where the emission signals are collected at different timepoints to reduce background or to view multiple agents with a single excitation and emission wavelength.^[59,114] Leading



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Figure 3. Interactions between light and biological tissue. A) The tissue penetration of light is wavelength-dependent. Longer wavelengths typically tend to exhibit deeper penetration depths in biological tissue. The light tissue penetration data in this diagram is based on a report by Ash et al. and indicates the approximate depth at which 1% of incident light energy of a 10 mm-wide laser in a skin model still exists.^[104,109] Note: The 808 and 980 nm lasers are commonly used excitation sources for upconversion nanoparticles. It should be noted the 808 and 980 nm laser depths are from a separate study and thus not directly comparable to the visible light penetration depths due to changes in experimental conditions.^[104,109] B) This wavelength-dependent light penetration depths typically exhibiting lower coefficients of absorbance (μ_a) and scattering (μ_s), i.e., lower extinction. Light absorbance occurs when light energy is transferred to the tissue upon irradiation. Light scattering occurs when light reflects off the tissue components, causing a reduction in the intensity of light continuing through the tissue. C) The near-infrared (NIR)-I optical window, enabling improved light-based imaging through NIR-based lasers. Within this optical window, 808 nm lasers typically exhibit deeper tissue penetration than 980 nm lasers due to the locally elevated absorbance of water in the 950–1,050 nm range. In contrast, wavelengths <700 nm are absorbed efficiently by tissue components, such as hemoglobin.^[110]

research has shown significant reductions in spectral lifetimes to as short as $2 \,\mu s$.^[115] Other groups have varied dopant ion concentrations to obtain spectral lifetimes as short as 25.6 μs to longer than 1 ms for specific hybrid UCNPs, enabling multiple probes to be imaged rapidly from a single excitation laser.^[62,116–118]

In addition to the luminescence lifetime tunability, UCNPs have been engineered to emit in the entire visible spectrum and beyond. Full-spectrum chromatic tuning of UCNPperovskite quantum dot (UCNP-PeQD) hybrids has been demonstrated with an increase in the emission wavelength range from what has been achieved with either UCNPs or PeQDs alone. These results indicate a synergistic effect between the two nanoparticle types.^[116] Initial attempts at full-color tuning involved selecting different activator lanthanide ions for the nanoparticle core to take advantage of their unique emission profiles.^[103] More recently, a study showed that a single architecture of multi-shell UCNPs can be designed to emit a full spectrum of colors in response to a single excitation wavelength with varying pulse lengths.^[119] Another approach to color-tuning involves silica-coating UCNPs while varying the porosity of the silica shell, enabling alteration of the dyes absorbed and tuning of the green-red spectrum.^[120]

Another study showed the fabrication of UCNPs with emission peaks at 540 and 654 nm. The ratio of the emission peaks was altered through excitation with 800, 980, or 1,530 nm lasers.^[121] A similar effect was achieved when changing the voltage applied to the UCNPs, resulting in a voltage-dependent red, green, or yellow emission upon laser excitation.^[122] Similarly, due to the multiple emission peaks of UCNPs, bandpass filters can be used to isolate the emission wavelength of specific luminescence peaks. For example, one study used the 455 nm emission peak of Yb/Tm particles for multiplexed super-resolution imaging.^[24]

Using multiple lanthanide dopants in the same UCNP or cluster of UCNPs has led to the design of particles with light-dependent action. In one study, UCNP clusters were synthesized with orthogonal activation capabilities, enabling 980 nm excitation for UCNPs tracking and 808 nm excitation to release a drug molecule from the UCNP cluster.^[123] Other researchers have found that various factors may affect emission properties, including sample concentration, temperature, surface modification, excitation power, and the types of lanthanides and their compositional ratios.^[61,124,125]

3. Bioimaging of Individual UCNPs

Ensemble imaging of UCNPs is often used for in vivo imaging applications, but considerable research aims to image and quantify individual nanoparticles. For example, recent advancements in elemental analysis enable the quantification of chemical compositions and reaction kinetics of individual colloidal nanoparticles.^[126,127] The spatiotemporal visualization of individual nanoparticles enables investigations into nanoparticle dynamics and the analysis of nanoparticle heterogeneity in structure or luminescence, impacting biological outcomes.^[128–130] The ideal UCNP exhibits a few critical properties at the individual particle level. First, the ideal UCNP is photostable, meaning that the signal does not noticeably decrease in intensity upon continued

excitation. Next, the ideal UCNP has a relatively high quantum yield, defined as the ratio of emitted upconversion photons and the photons absorbed by the upconversion system.^[131] Brightness and quantum yield are sometimes considered limitations of UCNPs in bioimaging experiments but may be balanced by other photophysical characteristics of UCNPs, including anti-Stokes luminescence with long emission lifetimes and photostability. Further, individual UCNPs can be resolved at sub-diffraction limit distances with super-resolution imaging at the single nanoparticle level.

3.1. Photostability

One of the most prominent advantages of using UCNPs in imaging applications is to excite with NIR lasers and capture light in the visible range. This anti-Stokes luminescence behavior reduces image background noise, sample autofluorescence, and tissue overheating. However, the NIR excitation is not unique to UCNPs as NIR fluorescent probes have also been developed to achieve similar advantages of NIR excitation. Downconverting NIR fluorescent probes enable impressive imaging results by utilizing detectors in the NIR-II range (900–1.700 nm).^[132–134] However, compared to quantum dots, carbon dots, and UCNPs, traditional fluorophores, including NIR dyes, tend to photobleach when excited for extended periods. Photobleaching is the process by which cycling between excited and ground states of the molecule causes irreversible damage to the molecular structure of the probes, reducing or eliminating a dye's ability to emit light when excited.^[135]

Quantum dots are another imaging probe that is commonly used in nanoparticle applications.^[63,136,137] However, quantum dots are sometimes less photostable than UCNPs and are often prone to luminescence intermittency, or photoblinking, during imaging experiments.^[34] UCNPs, in contrast, have been shown to exhibit strong, unchanged, and continuous signals for greater than one hour of uninterrupted excitation.^[22,43,138–140] In addition, the non-blinking, steady signal makes UCNPs an ideal contrast agent for experiments requiring imaging over extended periods, such as cellular uptake or (intracellular) nanoparticle tracking experiments.^[128] It is worth noting that when combining UCNPs with organic dyes for photodynamic therapy or imaging applications, the photostability of the UCNP-dye hybrid is typically limited by the photobleaching properties of the dye rather than the UCNP.

3.2. UCL Enhancement

Extended UCNP imaging experiments with high laser powers can lead to tissue overheating due to water exhibiting an absorption peak in the NIR range.^[75,141,142] One possible way to overcome this challenge is to engineer UCNPs with higher quantum yields to achieve enhanced luminescence intensities with lower laser excitation powers. Initial UCNP systems often resulted in quantum yields of <5%, whereas quantum dots and fluorescent probes can achieve >50% quantum yields, and recent carbon dots have quantum yields as high as 86%.^[5,142–144] Recent studies reported UCNPs quantum yields in the 5%–7% range.^[53,145] Jones et al. proposed an initial framework for standardizing quantum yield measurements to enable better comparability between studies.^[145] A common cause for the low UCL quantum yield is concentration quenching. UCNPs with high lanthanide dopant ion concentrations will transfer their excitation energy to the surface or nearby dopant ions instead of photon emission.^[8,146,147]

Additional research has shown reductions in luminescence intensity due to quenching, dissolution, and leaching of dopant ions when in solution, specifically aqueous solutions.[148] However, this effect may be overcome by intentionally selecting surface coatings.^[27,149] Increasing the lanthanide ion concentration can increase the emission intensity due to increased ion concentrations but does not necessarily increase quantum yield.^[42,56] A well-established approach to increasing upconversion emission intensity and quantum yield is adding a shell to the UCNP architecture, which often consists of the same material as the core without sensitizing and activating ions. One group found a 13 times increase in UCL intensity simply by adding a shell to the UCNPs.^[85] Other research found that shellmediated UCL enhancement varies depending on the core/shell material, with enhancements ranging from 5-167.7-fold enhancement.^[86,150] These enhancement quantities may vary between upconversion emission peaks and between different excitation laser power densities.^[141] Researchers have investigated the optimal thickness of the shell, which leads to an increase in UCL efficiency by increasing the distance between the luminescent core and potential luminescence quenching molecules around the particle, and results tend to point to an optimal shell thickness of $\approx 5 \text{ nm.}^{[131]}$

Other approaches to UCL enhancements have focused on engineering homogeneous UCNPs to maximize the number of ions in a single particle while maintaining the distance separating them to prevent cross-relaxation.^[121,151] Another luminescence enhancement is by creating dve-UCNP hybrids, selecting dyes with energy transfers that closely match the lanthanide, enabling efficient energy transition between the two. For example, an ATTO 542 dye was adsorbed onto the surface of erbiumdoped UCNPs and resulted in a 2-3 times increase in quantum yield compared to the as-synthesized particles.^[152] Other research achieved an 18 times increase in UCL intensity when the optimal amount of Ag₂Se quantum dots was added to quantum dot-UCNP composites.^[63,70] Dye-mediated UCL enhancement was found to increase luminescence by 283% when the sensitizer ytterbium was integrated into the shell of the UCNPs to further facilitate dye-core energy transfer.^[153]

Although not the focus of this review, it is important to note that numerous studies use upconversion-capable lanthanide nanoparticles for downconversion luminescence applications, often utilizing the NIR-II (1,000–1,700 nm) emissions in conjunction with 980 or 808 nm excitation lasers. These luminescence downconversion capabilities have been successfully applied to cell targeting/phototherapy,^[154,155] in vivo multiplexing,^[38,156–158] and imaging optimization.^[63,134] For more information on the topic, Yang and colleagues recently published a review covering NIR-II emitting lanthanide fluorescent probes.^[159]

In addition to the nanoparticles themselves, excitation lasers can be altered to enhance emission intensity in UCNP applications. For example, Yan et al. observed a 49% increase in UCL signal when ytterbium/erbium UCNPs were simultaneously excited with both 1,550 and 980 nm lasers compared to the summation of both intensities individually, potentially pointing at a synergistic effect in multi-laser excitation.^[103] Additionally, using a laser cavity in the excitation laser increased upconversion emission intensity by an order of magnitude.^[160] Wen et al. recently published a review focused on enhancing UCL quantum yields for further reading.^[8]

3.3. Super-Resolution Imaging

In addition to a consistent luminescence signal, imaging experiments to study interactions of individual nanoparticles require the ability to resolve individual particles near each other. In light microscopy, the resolution limit is determined by Rayleigh's criterion, stating that the minimum distance that can be resolved between two separate sources is governed by Equation (1)

$$R = 0.61 \times \lambda / n_{\rm a} \tag{1}$$

where *R* represents the lateral resolution limit, λ represents the objects' emission wavelength, and n_a is the numerical aperture of the objective lens.^[161] Other similar equations dictate the lateral resolution of optical imaging, such as Abbe and Sparrow resolution but only differ in the leading coefficient.^[162]

As a result, typical resolution limits in light microscopy are \approx 200 nm. One important note is, that as wavelength increases, so does the associated resolution limit. Additionally, the resolution limit of optical microscopy depends on the number of photons absorbed by the imaging probe due to the difference in emission intensity between the center and edges of the excitation laser, enabling multi-photon microscopy to achieve super-resolution images.^[23,163] This reduction divides the resolution limit by the square root of the number of photons involved in the upconversion process, providing inherently multi-photon UCNPs with increased utility in super-resolution imaging.^[23]

Recent research has developed other techniques aimed at super-resolution imaging. Improvements in resolution through different super-resolution techniques are visually portrayed in Figure 4. Numerous approaches for super-resolution have been developed, to select a method for a specific application, Frances-Soriano et al. commented and recommended a super-resolution techniques for different types of imaging experiments.^[164] One commonly used super-resolution technique is stimulated emission depletion microscopy (STED). In STED, an excitation laser is rapidly followed by a longer wavelength, doughnut-shaped deexcitation laser, reducing the signal from objects outside the doughnut's center, and providing improved resolution.^[165] Liu et al. applied STED to image UCNPs at resolutions as low as 28 nm by using a 980 nm excitation laser combined with an 808 nm STED laser to isolate the ${}^{3}H_{4} \rightarrow {}^{3}H_{6}$ transition, achieving the desired reduction in resolution (Figure 4A).^[43] Compared to the 4 ms pixel dwell time achieved by Liu et al., Peng et al. reported a 400-fold reduction in pixel dwell time to 10 µs in STED by increasing dopant ion concentrations while reaching a lateral resolution limit of 72 nm.^[166]

Alternatively to STED, Chen et al. used a single doughnut beam excitation laser to enable NIR–NIR excitation-emission of UCNPs through a $93\,\mu\text{m}$ tissue sample, achieving a lateral resolution of < 50 nm. This technique was termed "near-infrared emission saturation nanoscopy" (NIRES).^[67] Additionally, the authors found that UCNPs with lower lanthanide ion doping concentrations had lower resolution limits when compared to highly doped UCNPs at equivalent laser excitation powers.^[67] Chen further developed a technique requiring the same single doughnut-shaped excitation laser but instead using both 800 and 740 nm emissions from the UCNPs to decode images and achieve a resolution of 40 nm, or \approx 4% of the excitation wavelength.^[168] Similarly, the same research group used a Bessel beam, whose amplitude is defined by a first-order Bessel function, reporting an excitation power-dependent resolution of 37 nm and a resolution of 98 nm when imaging UCNPs at a depth of 56 µm through MCF7 tumor spheroids, which have a significantly higher density and thus scattering coefficients than typical tissue models.^[169]

Camillis et al. also demonstrated multiplexed imaging in another super-resolution technique, super-linear excitationemission microscopy (uSEE).^[24] Denkova et al. first reported this imaging technique in 2019 and relied on super-linear probes only being excited by the excitation laser's central, most intense portion (Figure 4B). The primary advantage of uSEE microscopy is not needing additional modifications to a traditional confocal laser scanning microscope or requiring exotic or complicated UCNP architectures (NaYF₄:20% Yb, 8% Tm). After optimizing experimental conditions, the authors reported halving the resolution limit in a fixed cell sample.^[167]

Another common super-resolution technique applied to UCNPs is structured illumination microscopy (SIM). SIM achieves super-resolution by using grates or filters to apply a patterned excitation to the sample. This pattern is then changed and applied to the sample again, and post-processing uses multiple images to better identify the particle locations and reduce resolution limits.^[170] Figure 4C shows one application of upconversion nonlinear structured illumination microscopy (u-NSIM), which combines the super-resolution capabilities of SIM with the NIR-excitation property of Yb/Tm UCNPs to achieve a resolution of around 130 nm as well as a clear resolution of 350 nm lines through a 51.5 µm thick section of liver tissue.^[51]

Multiplexing of imaging probes has further been realized through a similar SIM technique termed time-resolved structured illumination microscopy (TR-SIM), where altering the thickness of a migration layer separating the Nd³⁺ sensitizer layer and the Yb³⁺ core can alter the lifetime curve of the UCNPs. Using this technique, the authors could have three separate subsets of UCNPs that only varied by the size of their migration layer. They imaged these UCNPs with a single detector and single excitation laser while achieving a lateral resolution of 185 nm.^[25]

Other super-resolution techniques have taken advantage of photon avalanche mechanisms in upconversion, where a nanoparticle material has significantly stronger absorption in an excited state as opposed to ground state energy levels. Upon initial excitation, ions can occupy an excited energy state and through cross-relaxation then pair with a ground-state ion to create two intermediate-energy ions. Due to a high absorption at this intermediate excited state, these ions can then repeat this process, creating many intermediate and excited-state ions,



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Figure 4. Visualization of individual UCNPs through super-resolution techniques. A) Super-resolution stimulated emission depletion microscopy (STED) images of 40 nm UCNPs doped with NaYF₄ (20% Yb and 8% Tm). The authors reported a lateral resolution limit of 28 nm when using 13 nm UCNPs at an excitation power of 7.5 MW cm⁻² (scale bar = 500 nm).^[43] Adapted with permission.^[43] Copyright 2017, Nature. B) Upconversion super-linear excitation-emission microscopy (uSEE) can resolve NaYF₄ (20%Yb/8%Tm) UCNPs separated by \approx 200 nm. The authors reported lateral and axial resolutions of 184 & 390 nm, respectively, using the uSEE technique (scale bar = 200 nm).^[167] Reproduced with permission.^[160] C) Upconversion nonlinear structured illumination microscopy (U-NSIM) has been used to image NaYF₄ (20%Yb/4%Tm) UCNPs. The authors noted the ability to resolve induvial UCNPs separated by 161 nm (scale bar = 2 µm).^[51] Adapted with permission.^[51] Copyright 2020, American Chemical Society. D) Photon avalanche upconversion nanoparticles imaged at 828 (left) and 72 kW cm⁻² (right). At 76 kW cm⁻², Liang et al. achieved a full-width half maximum of 62 nm for a single particle. The projection on the right is a 10-point moving average of the relative pixel intensity of the line drawn through the particle.^[140] Reproduced with permission.^[160]

enabling a "photon avalanche" effect when falling back to the ground state.^[23,57,171] This mechanism is displayed in Figure 2. Researchers have developed UCNPs to absorb as many as 80 photons in the multi-photon process, leading to a proportional reduction through the multi-photon process with resolving capabilities at distances as short as 20 nm.^[23] These photon avalanche, energy-looping UCNPs often use a non-resonant excitation through a 1,064 nm laser to target the excited state energy absorption in thulium ions. A recent study imaged the same UCNPs with and without initiating the photon avalanche effect and showed a drastic reduction in the full-width half maximum of a single particle, from 217/216 to 81/65 nm for the long and short axes, respectively.^[46] However, a primary hurdle in superresolution photon avalanching is extended imaging periods. Because many excitations are required before the avalanche effect is able to occur, the pixel dwell times for images using photon avalanching often are in the 10-100 ms range, resulting in long imaging times and extended excitation laser exposure.[172] However, a recent publication has achieved photon avalanche pixel dwell times as low as 0.1 ms with resolutions of 71 nm. Simultaneous avalanching of praseodymium and ytterbium ions utilized the closely matched excited energy states of ytterbium and praseodymium to rapidly populate the atoms' excited states and reduce the time necessary to achieve the avalanching effect. Additionally, this technique was capable of 26th-order nonlinearity and of further transferring energy to thulium or holmium activator ions to achieve 46th-order nonlinearity.^[140] This technology was utilized to acquire super-resolution, single-excitation laser, single-particle images in HeLa cells.^[140]

Super-resolution technologies have been further used for UCNP characterization. Ren et al. use stochastic optical reconstruction microscopy (STORM) to distinguish 170 nm rod-shaped UCNPs with fluorescent probes solely on the ends of the nanoparticles or evenly distributed throughout the nanoparticle.^[173] STORM imaging excites a selection of optically resolvable fluorophores in the sample of interest by using multiple lasers to cycle emission sources between light and dark states. By continuing to excite a fraction of total fluorophores through multiple images, emission overlap does not occur, allowing for a more exact location of emitted light to be determined.^[174]

A potential drawback to many of these super-resolution techniques is the laser power needed to achieve the best resolution. For example, NIRES imaging used a laser power of 4 MW cm^{-2}



and, in achieving 28 nm resolution through STED, excitation lasers at powers of 0.66 MW cm⁻² and suppressive lasers with power as high as 9.75 MW cm^{-2} were used.^[43,67] When using the Bessel beam for resolving particles in spheroids, 8.9 MW cm⁻² of laser power was necessary.^[169] Photon avalanching may use reduced laser powers, for example, Liang et al. used a $76 \text{ kW} \text{ cm}^{-2}$ 852 nm laser to achieve resolutions as low as 62 nm (Figure 4D).^[140] However, further reductions in laser power are still required to prevent sample overheating. For comparison, Liu et al. found that a 4 kW cm^{-2} , 976 nm laser imaging through a 51.5 µm tissue slice resulted in a 3 °C temperature increase in the sample.^[51] Other articles have noted that laser powers in this range are not feasible for live-cell imaging experiments, and laser powers at 1 kW cm⁻² may lead to photodamaged cells.^[141] Nevertheless, regarding sample overheating, applying super-resolution techniques to imaging in cellular experiments will require more focus on reducing the laser power necessary to achieve super-resolution or using lower-laser power techniques such as u-NSIM alone combined with postprocessing methods such as Hessian deconvolution or artificial intelligence.^[66,168,175,176] A recent book chapter focusing exclusively on UCNPs and super-resolution imaging is available for further information on the topic.^[177]

4. Bioimaging in Live Cells

In living cell microscopy experiments, the desirable characteristics of nanoparticle bioimaging probes include non-cytotoxic behavior, the ability to image specific cellular compartments and organelles, and the ability to be used in tandem with other imaging probes. This section explores UCNPs' ability to fulfill these characteristics and highlights recent advances in cellular imaging.

4.1. Cytotoxicity

Two primary means of cell death could occur in UCNP imaging experiments: first, extended NIR laser exposure may reduce cell viability due to phototoxicity and overheating^[178]; second, the UCNPs themselves could induce cytotoxicity. Most research shows minimal UCNP-induced cytotoxicity when dosed with relevant concentrations. For example, Zhang et al. found that in clusters of Tm³⁺ and Er ³⁺ sensitized UCNPs, minimal cytotoxicity was observed in concentrations up to $500 \,\mu g \,m L^{-1}$. The cytotoxic effect of UCNPs has been found to vary based on surface coating: bare UCNPs resulted in elevated cytotoxicity when compared with silica- or cucurbit[7]uril- coated UCNPs, and cell line: HeLa and RAW 264.7 cells exhibited elevated cytotoxicity whereas EAhy 926 endothelial cells did not.^[179] Additionally, neither 980 nm nor 808 nm lasers with power densities of 2.5 W cm^{-2} for as long as 20 min caused a notable reduction in HeLa cell viability. However, at these levels, a nonsignificant downward trend between the controls, 10, and 20 min time points may point to reaching an upper threshold of laser power for this experiment.^[123]

Other studies have found no toxicity in HeLa cells irradiated for three 5 min on/off cycles with 980 mm laser power densities up to 5.8 W cm^{-2} .^[79] However, Levy et al. found HeLa cells did

not experience ruptured membranes, indicating phototoxic cell death, when exposed to 1,064 nm excitation at $10^6\,W\,cm^{-2}$ for over two hours. $^{[57]}$ Similar nanoparticles dosed in HeLa and Cal27 cells show a slight downward trend in cell viability with $\approx\!80\%$ viable cells after dosing with UCNPs at $1\,mg\,mL^{-1}.^{[180,181]}$ Numerous other studies engineered UCNPs for photodynamic therapy found minimal reduction upon dosage and a stronger reduction in cell viability following laser activation. $^{[182,183]}$

A possible method to account for sample overheating in experiments is using UCNPs capable of temperature sensing in physiological ranges through ratiometric sensing.^[13,184,185] Alternatively, a pulsed laser excitation, as opposed to continuous-wave laser excitation, was found to have a proportional impact on sample heating, meaning that using a 1:1 on/off pulsed laser would be expected to reduce sample heating by 50%.^[59] Increasing the time between image acquisition steps could have similar effects. In whole-body imaging, it has been shown that 808 nm lasers have lower impacts on tissue overheating than 980 nm lasers while also showing significantly deeper tissue penetration.^[101]

Additional concerns in the use of UCNPs could arise from the synthesis of these particles involving more hazardous, toxic reagents as compared to the synthesis of gold nanoparticles. For example, UCNP synthesis oftentimes requires 1-octadecene, a hazardous chemical, and lanthanide salts, many of which have limited study on their ecotoxic effects, resulting in a less-green synthesis protocol.^[186]

4.2. Surface Modification

An ideal nanoparticle-based bioimaging probe can be modified to target and enable observation of specific intracellular organelles, proteins, DNA strands, or other areas of interest. Briefly, UCNPs have been coated with DNA strands,^[76] antibodies,^[187] amine groups,^[188] cell membranes,^[73] and ligands/polymers/surface charges^[30,181,189–191] among others, and have been engineered to be activatable for imaging or release of loaded molecules (Table 1).^[180] For example, using Nile red dye derivative-modified UCNPs, iron ions were detected in cells at concentrations of 89.6 nM.^[192] Presumably, other metal ions of interest could be detected and quantified by similar strategies by finding dyes that selectively react with the targeted ion.

Dress et al. used lanthanide resonance energy transfer (LRET). In this technique, UCNP proximity is used to selectively excite a nearby luminophore to visualize the interaction between two mitochondrial matrix proteins Tom20 and Tom7.^[193] Zhan et al. used STED to achieve super-resolution cytoskeleton images by conjugating a secondary antibody to their UCNPs in HeLa cells.^[187] Through immunolabeling, UCNPs have been engineered to differentiate between cell types, enabling their application to distinguish between multiple cell types in heterogeneous cultures.^[94]

Nanoparticles are often labeled with external luminescent compounds to enable visualization in optical imaging. UCNPs exhibit intrinsic UCL, i.e., the UCNP itself is the source of the luminescence signal. This characteristic is advantageous compared to fluorescently tagged nanoparticles, where the fluorescent label could alter the surface chemistry of the nanoparticle and, in turn, alter the downstream nanoparticle–biology and nanoparticle–cell interactions.^[194,195] Being the luminescent label themselves, UCNPs enable label-free nanoparticle tracking compared to other fluorescent tagging techniques, and thus a wider range of potential experiments.

4.3. Multiplexed Imaging

Multiplexing is an attractive feature of nanoparticles in cellular experiments. Ideally, the multiplexing nanoparticles would be physically and functionally identical within the cellular environment and only differ in their end target and their emission upon excitation, enabling single-laser excitation if necessary and preventing physicochemical differences between nanoparticles to alter cellular fate.^[130] Compared to fluorescent probes, which require multiple excitations, past work has shown that UCNPs can have tunable emission lifetimes by altering the size of migration shells, customizable upconversion emission colors through changing laser pulse, or both occurring in the same particle.^[25,114,119] A summary of these techniques can be seen in **Figure 5**.

Figure 5A depicts a lifetime-based multiplexing technique where the thickness of an inert inner shell affects the time necessary for the excited outer shell electrons in the sensitizer to transfer energy to the activator, enabling differentiation of UCNPs by taking numerous images in succession and identifying which timeframes each UCNP appears in. An additional technique was developed to distinguish two nanoparticle populations using the doping concentration-dependent laser-power threshold of UCNP emissions. In this article, Camillis et al. noted the highly sloped linear relationship between the 455 nm emission in thulium-doped UCNPs and the excitation laser intensity. Exploiting this property, 8% and 16% thuliumdoped UCNPs were distinguished by isolating the 455 nm emission through an 808 nm co-excitation laser. To distinguish particles, images were taken with two different laser powers, one below the excitation detection threshold for the 16% particles but high enough to view the 8% particles at \approx 10% of the maximum emission, and the other with sufficient laser power to view the 16% particles at \approx 10% emission and the 8% particles at maximum emission power (Figure 5B).^[24] A similar, single-excitation laser technique has been developed in thulium-doped UCNPs, where altering laser excitation power makes the 1%, 8%, or both sets of UCNPs visible.[128]

Another promising multiplexing application involves frequency multiplexing. For example, two ytterbium-based UCNPs were made, one with a holmium activator and the other using erbium. When imaged, both particles were visible during 977 nm laser excitation, however, co-excitation with a 790 nm laser selectively excited the erbium particles, whereas co-excitation with a 750 nm selectively excited the holmium particles (Figure 5C).^[196]

A final potential multiplexing approach with a limited demonstration in the current literature is activator-emissionbased multiplexing. Doping with different activator ions leads to different upconversion emission spectra, enabling the engineering of UCNPs to have different emissions from a single www.adpr-journal.com

excitation laser.^[39,197] By imaging with detectors at each emission peak, the proportional intensities could be used to determine the identity of particles. By coating UCNPs with an identical NaYF₄ core, these particles would have similar surface chemistries and provide an additional avenue for multiplexing of UCNPs with a single-laser imaging setup. In addition to lifetime multiplexing, this activator-based multiplexing technique has further been demonstrated in vivo to identify different nanoparticle populations or differentiate tumor cell lines in mice.^[53,94]

In summary, multiplexing techniques have been developed for the single-laser-single detector (lifetime), multi-laser-single detector (frequency and excitation power), and single-laser-multi detector (activator) imaging setups. These techniques have been employed in systems requiring multiplexing of two or three UCNP populations. Future work may focus on increasing the number of distinguishable UCNPs populations or increasing the accuracy at which these populations can be distinguished. One potential avenue could be in a combination of multiplexing techniques. For example, Liu et al. developed seven UCNPs populations (τ^2 -1 to τ^2 -7) with increasing spectral lifetimes and achieved \approx 70% accuracy in distinguishing UCNPs populations and 93% when using three populations. Most misidentifications in the seven-UCNPs population method occurred due to difficulty distinguishing particles with nearby spectral lifetimes (i.e., $\tau^2 - 2$ and $\tau^2 - 1/\tau^2 - 3$).^[25] However, if combined with a frequency encoding method, where populations 1, 3, 5, and 7 were holmium-based UCNPs and populations 2, 4, and 6 were erbium-based UCNPs, the largest source of error could be eliminated, increasing the accuracy of identifying UCNPs.^[196] Alternatively, within cellular imaging, using UCNPs in conjunction with downconverting fluorescent probes to stain the nucleus, cell membranes, or other cellular compartments of interest can reduce the number of multiplexed channels needed for an experiment.

4.4. Cellular Actions

In addition to targeting cellular structures and multiplexing, UCNPs have been engineered to enact cellular functions. One particularly interesting study found that by using azobenzenebased 808 nm laser-activated caps, a knockdown siRNA strand targeting a specific gene used to enhance therapeutic efficacy could be released on command from a UCNP cluster.^[180] Further developing this technique could lead to on-demand gene expression/inhibition or release of intracellular proteins in addition to the photodynamic therapy (PDT) effects explored in this study. Other gene-editing approaches focused on using UCNPs and CRISPR-Cas9 to achieve spatiotemporally activated gene editing in vitro and in vivo.^[20] Similarly, UCNPs with a mesoporous silica and amine shell were loaded with DNA to examine transfection and DNA delivery to primary rat heart cell lines.^[188]

The upconversion emissions of UCNPs have further been engineered to stimulate in vivo neural cells through the ChR2 receptor, triggering dopamine release, silencing seizures, or recalling memories.^[198] Similar results could be expected in cell culture experiments and have been used specifically with neural cells to dictate neural differentiation of induced pluripotent stem cells in vitro utilizing an 808 nm laser-activated conformational



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Figure 5. Potential approaches for UCNPs-based multiplexing in cellular imaging. A) Wang et al. demonstrated UCL lifetime engineering to control the UCNPs' emission lifetimes through nanoparticle design (scale bars = $2 \mu m$).^[25] The engineered UCNPs can be detected and identified by their characteristic UCL lifetimes in selected timepoint images, potentially enabling UCNPs emission lifetime multiplexing for cellular imaging. Reproduced with permission.^[25] Copyright 2022, The Royal Society of Chemistry. B) Excitation power multiplexing of UCNPs exploits changes in lanthanide ion dopant concentrations of various UCNPs to alter the power thresholds necessary for UCNP detection. Combined with the super-linear relationship between excitation power and upconversion emission intensity, lower doped UCNPs are visible at lower excitation powers and saturate at similar laser powers where the higher doped UCNPs begin to be visible, enabling the distinction of the two UCNPs populations for potential applications in cellular imaging (scale bar = $1 \mu m$).^[24] Reproduced with permission.^[24] Copyright 2020, The Royal Society of Chemistry. C) Vosch et al. demonstrated a frequency-encoding method to enable UCNP multiplex imaging (scale bar = $10 \mu m$). Co-excitation with secondary lasers targeted specific energy transitions for enhanced absorption by either holmium or erbium.^[196] Adapted with permission.^[196] Copyright 2021, American Chemical Society.

change in the shell resulting in the release of retinoic acid from the particle. $^{\left[199\right] }$

A sizable amount of nanoparticle research is focused on nanoparticle trafficking, or studying how nanoparticles are taken into cells, where they travel once inside cells, and the end fate of nanoparticles interacting with cells. Current methods to monitor nanoparticle trafficking in live cells often involve fluorescent tagging of the particles of interest. Recent research has used naked nanoparticles and reflected light to visualize cell–particle interactions.^[200] As stated previously, fluorescent surface modifications can alter the pathway a particle may take inside the cell compared to the label-free particle.^[194] UCNPs circumvent this problem by their core being the source of luminescence, enabling the engineered surface chemistry to be responsible for the particles' cellular fate. To that aim, UCNPs, when combined with methods for inhibiting certain uptake pathways, have been used to determine how nanostructures interact with cellular membranes and enter cells.^[181] Additionally, upon entering the cellular environment, Wang et al. developed a technique to track and map the movements of individual 40 nm UCNPs as they move through the cell over the course of multiple minutes.^[128] Combining these technologies with UCNPs coated with silica, lipid membranes, or gold could result in particles with identical surface properties to current silica, lipid, or gold nanoparticles with UCL capabilities enabling long-term uptake and trafficking observation.^[86,201–203]



5. Bioimaging in Whole Animals

For animal-level imaging experiments, contrast agents based on UCNPs should exhibit desirable biocompatibility, detection through applicable tissue depths, prolonged circulation times in the bloodstream, and not produce body-level toxicity or other cytotoxic effects. Additionally, being modifiable to target specific organs, tumors, or tissues, UCNPs enable a broader range of applications, including assisted surgery and/or targeted therapies. On this level, much research has been conducted to engineer UCNPs better suited to fit these criteria. A summary of selected publications regarding whole-body and tissue imaging of UCNPs can be found in Table 1. The table explains the architecture, application, excitation, and upconversion emissions of the UCNPs used in the corresponding publications involving tissue/whole-body imaging, whereas the imaging depth reported may be from a different portion of the corresponding publication if the authors specifically investigated the depth at which their UCNPs were visible. Table 1 provides a representative, non-exhaustive list of UCNP bioimaging applications in tissues and invivo applications sorted by the article's aim and, to increase ease of organization, nanoparticle size. Studies falling under multiple table categories are listed in each applicable section.

5.1. Biocompatibility & Biodistribution

Poly(ethylene) glycol (PEG) is a general nanoparticle surface modification for extending circulation times of nanoparticles in vivo by reducing protein adsorption and lowering interactions of PEGylated nanomaterials with organs and cells of the mononuclear phagocyte system.^[80,84,92] However, recent studies have shown the potential for PEG coatings to cause unwanted immune side effects. As the details of PEG-mediated toxicity are outside the scope of this article, the authors recommend a review that was recently published by our research group covering PEG-mediated nanoparticle toxicity and other potential toxic effects of nanoparticles in animals and humans.^[204]

Alternative surface coatings that have been reported include colominic acid, which was demonstrated to have a three times longer circulation time than PEGylated particles; or heparosan, a nonimmunogenic natural polysaccharide shown to reduce protein adsorption as efficiently as PEG; or through modifying UCNP surfaces with DNA or proteins.^[78,205,206] An additional method to avoid immunologic side effects involves red-blood-cell membrane-coated UCNPs, which were found to allow "virtually no proteins" to adsorb to the surface of the particles.^[29] Other work has successfully coated nanoparticles with cancer cell membrane proteins to achieve similar results.^[73,77]

Ideally, the UCNPs would be eliminated from the body system following use for imaging applications, and organ distribution would be predictable and well-documented.^[207,208] A significant advancement towards these aims was published by Peng et al. in 2020, where UCNPs with a potassium heptafluozirconate core, viewable 7 mm beneath a mouse's skin at 1 W cm⁻² excitation power, were engineered to be biodegradable in aqueous solution. These UCNPs showed a total loss of luminescence signal and clearance from the body within 6 h and showed a reduced

degradation rate in the mildly acidic tumor microenvironment.^[72] A few key studies have further investigated the pharmacokinetics and biodistribution of UCNPs following administration in mice. In one study, UCNP-maleic anhydride/1-octadecene copolymer-HER2 targeting molecule nanoparticles were injected at 12.5 $\mu g \, g^{-1}$ and showed a substantial $\approx 20\%$ reduction in circulating concentration between 2 and 5 min following injection. The authors noted little to no accumulation in skin or muscles, with most accumulation occurring in the liver and spleen and a relatively low amount of UCNPs in the kidneys. Finally, the authors noted no adverse effects experienced by the mice aside from a brief, slight decrease in total leukocyte count.^[81]

Another study orally fed mice NaGdF₄:18% Yb/2% Er UCNPs coated with PEG and imaged UCL intensity for the mice's stomach, large intestine, small intestine, heart, lungs, liver, and kidney and did not find a noticeable signal outside of the digestive tract. The UCNPs remained in the digestive tract for 5 days following a dosage of $500 \ \mu g \ g^{-1}$.^[99] This is presumably due to the UCNPs never leaving the digestive tract and was supported by findings showing that even 5 nm UCNPs could not escape the digestive tract and enter the bloodstream or surrounding tissue.^[99] A few other studies that evaluated the biodistribution of UCNPs are available in the literature.^[73,79,92,93]

5.2. Imaging Depth

An additional constraint for bioimaging experiments involving UCNPs is the need for light to penetrate the body deep enough to activate the particles and for the light emitted from the UCNPs to be detectable. Table 1 lists imaging depths for a recent selection of invivo bioimaging experiments. Notably, typical laser power densities for these experiments range from \approx 100–10 W cm⁻² and can be used to image large numbers of UCNPs at maximum depths of $\approx 1 \text{ cm}$ below the surface of the mouse skin. Ideally, UCL imaging experiments should be able to image UCNPs at applicable depths, using laser powers deemed safe for dermal exposure, or $0.73 \,\mathrm{W \, cm^{-2}}$ for a 980 nm laser, according to the American National Standards Institute.^[55] A necessary clarification to make here is, regarding these in vivo bioimaging experiments, the imaging completed is often not resolving individual UCNPs at the listed depth but instead locating UCNP ensembles in the body. However, it should further be noted that at a whole-body level, resolving individual UCNPs may be less important than during invitro studies. For example, brain vasculature was accurately mapped at 400 µm through mouse brain tissue using a 980 nm 1 µmdiameter excitation laser with a power of 1.7 mW, which was noted to be 800,000 times lower than the power needed to activate and image FITC, a common fluorescent dye, at the same depth. This study did not track individual UCNP movement as opposed to observing UCL throughout the vasculature during imaging due to large quantities of UCNPs.^[61]

Analogous studies aiming to map mouse brain vasculature used 980 nm laser excitation with a laser power of 20 mW to reach imaging depths of 1 mm when combined with post-image deconvolution methods.^[66] To increase imaging depth in these experiments, one possible strategy would be to focus on tuning



emissions to be in the NIR-I (700–1,000 nm), NIR-II (1,000–1,350 nm), or NIR-III (1,550–1,870 nm) optical windows through NIR–NIR imaging, as the upconversion emission light is also subject to tissue attenuation.^[209] This approach has been shown by multiple studies, which have found detection depths of UCNPs for 800, 660, and 540 nm emissions to be 8 and 7.7, 4 and 5, and 2 and 2.3 mm, respectively.^[76,93] This result indicates that longer wavelengths can penetrate deeper into the tissue. By using NIR–NIR excitation/emission, the upconversion emission signal may also provide visualization of UCNPs further from the surface.

Another simple modification is using an 800 or 808 nm excitation laser instead of a 980 nm laser. This benefit is typically attributed to the ≈ 10 times higher absorbance of Nd³⁺ at 808 nm than Yb^{3+} at 980 nm and the ${\approx}25$ times higher absorbance of 980 nm light than 808 nm light in water. However, it is worth noting that tissue exhibits an increase in light scattering when moving from 980 to 808 nm.^[210] However, 808 nm excitation would require upconversion into the visible range, preventing NIR-NIR excitation-emission. Wiesholler et al. highlighted the increased performance of 808 nm excitation at deeper wavelengths but noted the significant impact of closely matching excitation lasers and absorption spectra for optimal imaging depth, noting a \approx 2.5-fold increase in photon absorption with their Nd³⁺ UCNPs when switching from an 805 nm to a 794 nm excitation laser.^[210] Liu et al. conducted experiments on the comparable performance of 800 and 980 nm excitation lasers. They found that, through raw chicken, the 980 nm laser was detected 0.2 cm beneath the surface, whereas the 800 nm laser was detected 2.5 cm beneath the tissue surface, both at a laser power density of 0.5 W cm^{-2} . Additionally, the 808 nm laser showed lower tissue overheating than the 980 nm laser.^[101]

Although less frequently, excitation lasers with wavelengths longer than 1,000 nm have been investigated for their utility in the bioimaging of UCNPs containing a wide range of lanthanide ions.^[41] Of potential interest are data reported by Wang et al. showing a 7.1- and 2.1-fold enhancement in spatial imaging resolution through a 3.5 mm tissue phantom for a 1,550 nm excitation laser when compared to 1,064 and 1,344 nm lasers, respectively.^[211] Nevertheless, potentially the greatest room for improvement regarding imaging depth is an increase in UCNP quantum yield. As quantum yield is defined as the ratio of absorbed to emitted photons, a quantum yield increase would lead to stronger emissions from identical non-toxic excitation powers, increasing the amount of tissue through which the UCNPs could be detected.^[212] For example, the UCNPs used by Liu et al. to achieve detectable UCL at a depth of 2.5 cm had a reported relatively low quantum yield of 0.75%.^[101]

Other techniques and results used to increase imaging depth include: 1) an \approx 3 times increase in penetration depth when increasing 808 nm laser pulse frequency from 500 Hz to 71.4 MHz^[213]; 2) an increase in signal-noise ratio through the use of highly doped (60% sensitizer/40% activator) UCNPs as opposed to UCNPs with a more common, i.e., lower lanthanide ion dopant concentration of 20% sensitizer and 2% activator^[55]; 3) a 3% increase in light intensity at 5 mm tissue depth using a high-frequency ultrasound to reduce scattering of light through the tissue^[214]; 4) or increasing the beamwidth of excitation lasers, where the increase from 1 to 10 mm lead to a penetration

increase of nearly 100% before further plateauing beyond $10 \text{ mm.}^{[104]}$

Applications that focus less on imaging and more on enacting photo-effects on nanoparticles in vivo, such as photodynamic therapy (PDT), or light-based activation of a desired effect, also focus on measuring penetration depth but focus less on emission detection. For example, Chen et al. showed that UCNPs emitting blue light could activate neurons at depths of up to 4.5 mm using a 2 W 980 nm laser and a calculated quantum yield of 2.5%.^[198] The same study modeled laser excitation and upconversion emission intensity at different depths through the brain tissue.^[198] One PDT application noted 60%–70% death of targeted cells at a depth of 1 cm by using NIR-stimulated upconversion emission from UCNPs to activate KillerRed, a green-light excited reactive oxygen species (ROS) generator.^[215] Other UCNP-based PDT applications are summarized in Table 1.

5.3. In Vivo Targeting

As shown previously, UCNPs have been engineered with surface modifications ranging from antibodies for immune-targeting to specific functional groups and tailoring UCNP surface charges for specific applications. These surface modifications have been applied to multiple in vivo targeting applications in systematic and locally injected UCNPs. Micelles with antibodies targeting a common membrane protein of pancreatic cancer cells were engineered and found to have an elevated targeting efficacy compared to non-targeted UCNPs.^[92] Seok et al. conjugated a breast cancer-specific antibody and colon cancer-specific peptide to two UCNP types with identical excitations but distinguishable upconversion emission wavelengths to successfully identify the location of two different cancer types in systemically administered UCNPs showing a threefold increase in accumulation when compared to bare UCNPs.^[94]

UCNPs administered in vivo have successfully passed the blood–brain barrier and delivered anti-cancerous effects to glioblastoma cells through angiopep-2, increasing the median survival time of the mouse treatment group against the control.^[91] Further research used a similar approach but added an 808 nm induced generation of reactive oxygen, enabling the on-demand endolysosomal escape of UCNPs in glioblastoma cells.^[88] Additionally, folic acid conjugated UCNPs enhanced targeting capabilities against breast cancer tumors grafted into mice.^[29] Other strategies involved targeting specific miRNA sequences in vivo to target cancer cells, potentially expanding targeting abilities for cancers or diseases with currently unknown protein elements.^[52]

The primary concern with enhanced targeting of tumors, organs, or biomolecules in whole-body systems is a moderately low increase in upconversion signal, and usually, as in the case of the folic acid conjugation, showing an approximate 3 times increase in targeting efficiency between the non-targeting and targeted UCNPs.^[29] This is a common issue faced in nanoparticle targeting, specifically within tumor targeting, as a median value of only 0.7% of injected nanoparticles reaching tumors and cancer cells is expected.^[216–219] A more in-depth review of nanoparticle-tumor delivery strategies was recently published by Sheth et al.^[220]



6. Applications of UCNPs in Multimodal Bioimaging

Long-term goals for the field of UCL bioimaging may include translating the method into clinical settings. One potential application could be in imaging-guided surgery. UCNPs provide real-time signals and can be designed to have upconversion emissions strong enough to be visible to the naked eye.^[56] This study by Shen et al. achieved naked-eye visible UCNPs for surgical resection through a 15 times increase in UCL intensity by doping the CaF₂ based UCNPs with a high concentration (98%) of Yb³ sensitizer ions. As opposed to repeatedly referring to ultrasound or MRI screens, upconversion light emission from targeted tissue could improve surgical accuracy and operating time. Another study investigated residual tumor mass following surgery and supported this idea, using 980 nm excitation and 545 nm emission UCNPs to target and illuminate ovarian cancer cells. Visual resection was found to leave $\approx 10.7\%$ of tumor mass following surgical removal whereas the UCNP-aided design reduced the residual tumor mass to 0.2%.[221]

UCNPs could further open new opportunities in clinical imaging that are currently unachievable with X-ray, magnetic resonance imaging (MRI), positron emission tomography (PET), and ultrasound techniques. Two examples were shown by utilizing UCNPs to improve surgical accuracy: the potential for molecular and protein targeting; and instant, visual feedback of optical signal. A third avenue for improvement in clinical bioimaging enabled through UCNPs is multimodal bioimaging. Multimodal bioimaging involves the combination of multiple bioimaging techniques, or in this case, UCL with other bioimaging techniques. With slight modifications, UCNP activity and localization can be and have been shown to be visualized with X-ray/computed tomography (CT), photoacoustic imaging (PAI), MRI, and single-photon emission computed tomography (SPECT). Examples of each of these applications and sample images are shown in Figure 6.

A few examples of different UCNPs types used in X-ray/CT imaging include BiF₃:20% Yb³⁺/ 2% Er³⁺ (Figure 6 bottom right),^[103] BaYF₅:20% Yb³⁺/ 2% Er³⁺/ Bi³⁺,^[223] NaGdF4:18% Yb³⁺/ 2% Er³⁺,^[99] 125 I-labeled NaYF₄:Yb³⁺/Er³⁺,^[80] and NaYF₄:Yb/Tm with a NaGdF₄:Yb shell functionalized with cyanamide and gold nanocrystals,^[79] among others. By developing a CT/UCNP multimodal bioimaging technique, X-rays could be used to overcome depth limitations or locating particles in the body before the targeting, multiplexing, and luminescent imaging UCNP capabilities could provide further information from a given imaging experiment. X-ray stimulation has been used to activate photodynamic therapy as well, applying this technique to UCNPs could enable PDT applications throughout the body, overcoming depth limitations of NIR light.^[224] Furthermore, CT values have been found to increase linearly as a function of UCNP concentration enabling at least a semi-quantitative signal.^[79] Similar linear relationships between localized UCNP and gadolinium ion concentration and signal intensity exist in MRI.^[101,225,226] Regarding MRI, several published studies use gadolinium ions dopants as the MRI-sensitive portion of the UCNPs. Although yttrium ions may be suitable for MR imaging, gadolinium is commonly used in MR imaging, making

gadolinium-based UCNPs an attractive contrast agent for MR-UCL bimodal imaging.^[227] For a proof-of-concept, the top right corner of Figure 6 shows elevated signal strength in a mouse abdominal tumor following the injection of UCNPs with a gadolinium-based shell. Liu et al. used a direct intratumoral injection of gadolinium-based UCNPs to show invivo contrast enhancement of MR signal in a mouse liver.^[101] Additional research used gadolinium-doped UCNPs combined with a red blood cell membrane coating and folic acid ligands to enhance nanoparticle localization in breast cancer tumor-grafted mice.^[226] Li et al. used a gadolinium-based UCNP core and peptide ligands to monitor accumulating nanoparticles in HCT 116 colon cancer cells.^[86] Gadolinium particles can also be used in trimodal CT/ MRI/UCL imaging.^[228] One potential drawback in MRI-UCNP bimodal imaging could be the extent of nanoparticle delivery efficiency, as most in vitro UCNP-MRI experiments showed limits of UCNP detection when gadolinium concentrations were in the single µM range.^[225,229] Additionally, research into erbium-based UCNPs showed a reduction in UCL intensity with gadolinium instead of yttrium particle cores, potentially hindering UCL/MR multimodal imaging.^[150] However, because the increasing magnetic field strength currently used in MRI is safe to use in humans and can increase the signal-to-noise ratio, future developments will provide lower detection limits in vivo applications.^[230]

Another prevalent pair of imaging techniques are PET and SPECT. A sample SPECT image of radiolabeled UCNPs can be found in the top left corner of Figure 6. UCNP experiments designed for PET/SPECT imaging have further been used in UCL, CT, MRI, and PAI.^[31] However, the key difference between PET/SPECT and other imaging modalities previously discussed is PET/SPECT requires the attachment of a radioactive tracer to visualize the uptake of molecules of interest into a tissue, which may take additional preparation, but enables more accurate quantification of nanoparticles, especially in organs located deep inside the body.^[231] For example, Kostiv et al. published two papers on multimodal imaging with 125 I-labeled NaGdF₄ core nanoparticles and used a neridronate linker to attach ¹²⁵I to the UCNPs. These PEG-labeled UCNPs enabled unmodified trimodal imaging of CT, SPECT, and MRI and monitoring of UCNP biodistribution for up to 14 days following injection in mice.^[80,229] Whereas UCNP-radiolabeling techniques may alter surface chemistry and, in turn, cellular interactions, one study developed a technique involving a two-part dosing process, where the initial red blood cell membrane-coated particles are first dosed in the mouse followed by a dose of fluorine-18 engineered to selectively bind the particles in vivo through a click chemistry process.^[226] This approach allowed for cellular uptake and biodistribution as well as UCL/MR imaging to occur before radiotracers entered the system, which may better portray the expected activity of the UCNPs in vivo.

Finally, UCNPs show potential utility in PAI. PAI is based on the photoacoustic effect, where nanosecond pulsed light absorption results in the formation of sound waves from the area of interest, which are detectable and can be mapped into an image, as seen in the bottom left corner of Figure 6. As opposed to other optical imaging techniques, PAI's main advantage is reducing the optical scattering of emission signals, enabling higher resolution in relatively deep biological tissue.^[232] One potential application of PAI in multimodal UCNP imaging was shown



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Figure 6. UCNPs enable multimodal bioimaging and in vivo imaging. UCNPs can be designed for multimodal imaging, including but not limited to X-Ray/computed tomography (CT) imaging, PAI, single-photon emission computed tomography (SPECT), and magnetic resonance imaging (MRI). The center circle shows the substantial increase in UCL signal following local administration of UCNPs in mice.^[52] Reproduced with permission.^[52] Copyright 2019, American Chemical Society. T₁-MRI images of tumor-bearing mice following injection of PEGylated NaYF₄:Yb,Er@NaGdF₄ encapsulated in tumor-targeting antibody-conjugated micelles (top right).^[92] Reproduced with permission.^[92] Reproduced with permission.^[192] Copyright 2016, American Chemical Society. CT imaging following subcutaneous injection of BiF₃:Yb,Er UCNPs (bottom right).^[103] Reproduced with permission.^[114] Copyright 2017, American Chemical Society. Photoacoustic in vivo images of gold nanorod dimer-UCNP-chlorin e6 injected via tail injection accumulating in mouse-grafted HeLa cell tumors (bottom left).^[95] Reproduced with permission.^[128] Copyright 2018, American Chemical Society. SPECT imaging monitoring ¹⁵³Sm radiolabeled UCNP accumulation in specific organs following injection in mice (top left).^[222] Reproduced with permission.^[223] Copyright 2013, Elsevier.

by observing tumor angiogenesis and UCNP accumulation over a period of time through PAI and observing full-body distribution using UCL imaging.^[101] Another notable advancement in the field was realized by Wang et al., where UCNP excitation by an 800 nm laser was used to excite fluorescent dyes through the tissue to use PAI instead of UCL imaging of dyes in solution. This approach, combined with UCNP emission tuning through excitation laser modulation, could lead to multiple fluorescent probes being imaged through depths >1 cm.^[233] Reducing background noise in PAI of UCNPs, and thus reducing limits of detection, was achieved by developing reversible photoswitching probes, where NIR light could switch the probe on or off. This allowed for the imaging of a signal-off background leading to an increase in signal-to-noise ratio and detection of 10⁴ cancer cells following implantation in live mice.^[234]

With regards to photodynamic therapy, PAI has been developed to monitor localization as well as deliver indocyanine green dye to cells, effectively reducing viability.^[102] Additionally, UCNPs engineered to be injected in microbubbles were found to release compounds of interest upon exposure to ultrasonic waves and, in turn, induce cytotoxicity following confirmed accumulation in the tissue of interest.^[82] Through a similar technique, PAI could be used to achieve a targeted therapeutic effect. Other research has used azobenzene-polymers to enhance PAI contrast and then used PAI UCNPs to detect and diagnose deep tissue diseases before using the more rapid feedback of NIR-II emissions from the same UCNPs to provide accurate surgical guidance during operation.^[235] Additional research has combined UCNPs with PAI to monitor the concentration of peroxynitrite, a biomarker for hepatotoxicity, to noninvasively monitor drug-induced liver damage in mice.^[236]

7. Limitations and Obstacles in Advancing UCNP Bioimaging

With regards to in vitro imaging experiments, UCNPs are highly adept to fulfill the requirements of most current bioimaging research. The literature reviewed in this work has shown UCNPs are not only strong candidates for bioimaging experiments in comparison to other commonly used modalities today, but also show strong promise and potential as they are further developed for use in bioimaging. UCNPs are presently capable of a wide, continuously expanding array of surface coatings to target intracellular targets and enable light-mediated actions at the subcellular level. Additionally, the combination of tunability and customization, self-luminescence, narrow emission bands, and super-resolution microscopy capabilities while using the NIR-range upconversion is useful for researchers across biomedical disciplines and provides a flexible bioimaging toolbox that is unique compared to other bioimaging modalities. Although presently capable, UCNPs as an emerging class of contrast agents for various imaging modalities may be further improved.

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For example, a limitation in live-cell experiments is achieving super-resolution imaging in living cells while still resolving individual UCNPs from other agents or fluorescent dyes used in tandem without exposure to harm-inducing laser powers. Efforts to reduce the necessary excitation power densities may enable super-resolution imaging of single UCNPs in intact live cells without affecting the biological functions of the cells.

At the whole tissue and animal levels, UCNPs appear to be very well-positioned for bioimaging experiments. The particles are capable of in vivo targeting, multimodal imaging, and multiplexed imaging of particle populations. As with most optical imaging modalities, the primary challenge is the limited tissue penetration depth of light signal due to light extinction. Noninvasive optical imaging is, therefore, often limited to the dermal layer. More effective UCNP technologies with increased tissue penetration depth may enable the accurate non-invasive imaging of smaller and deeper located organs or tissues of interest with improved spatiotemporal resolution. This advancement may require further enhancement of UCNP quantum yields. Furthermore, UCNP targeting capabilities have shown relatively low nanoparticle delivery efficiencies to specific organs and tissues in mice upon systemic administration.^[237] As researchers across the field of nanomedicine improve nanoparticle in vivo delivery efficiencies, these emerging technologies may be adaptable to the UCNP design. The increased delivery will advance UCNP applications in bioimaging, phototherapy, and targeted molecule release by enabling more effective tissue and cell targeting efficiencies as particles may accumulate more specifically through immunolabeling and selectively activated through the localized application of excitation light.

To translate UCNPs technologies into safe and effective real-world applications and clinical use, additional studies are needed to further assess the fate of administered UCNPs in the body. More comprehensive toxicological studies are necessary to evaluate how UCNPs degrade in the body or how these nanoparticles are excreted and eliminated from the body to facilitate the development and clinical translation of UCNP-based imaging technologies.

8. Conclusions

UCNPs show promise in bioimaging applications. UCNPs have been used in numerous cellular imaging experiments due to their inherent utility for UCL-based visualization and further in creating desired cellular actions upon excitation light activation. Super-resolution imaging of individual UCNPs has been demonstrated in the recent literature to track spatiotemporal distributions. UCNPs are uniquely positioned to enable targeted theranostic functions due to their ability to enact light-dependent localized effects. Potential clinical applications highlight the use of UCNPs for multimodal bioimaging, including combinations of X-ray, PET/SPECT, PAI, and MRI applications. Further research in the combinations of these bioimaging methods will enable single particle-type combinations of deep tissue imaging and increasingly targeted therapeutics as well as disease diagnosis and surgical guidance, among other applications.

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Conflict of Interest

The authors declare no conflict of interest.

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