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**BREAKING THE DEPTH-RESOLUTION TRADE-OFF IN
NONLINEAR MICROSCOPY: ADAPTIVE OPTICS AND
COMPUTATIONAL SUPER-RESOLUTION WITH
UPCONVERSION NANOPARTICLES**

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**Breaking the Depth-Resolution Trade-off in Nonlinear
Microscopy: Adaptive Optics and Computational Super-
Resolution with Upconversion Nanoparticles**

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A thesis submitted in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

July 2025

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ABSTRACT

Nonlinear optical microscopy (NLOM), typified by multiphoton microscopy, provides intrinsic optical sectioning for deep-tissue imaging but typically requires high-intensity pulsed excitation that induces phototoxicity and photobleaching. Lanthanide-doped upconversion nanoparticles (UCNPs) effectively overcome these constraints. Upon continuous-wave (CW) near-infrared (NIR) excitation, they sequentially absorb multiple photons and emit higher-energy NIR or visible fluorescence, thereby enabling high-resolution, high signal-to-noise ratio and multicolor imaging with substantially reduced excitation requirements and minimal photodamage. Furthermore, their exceptional photostability effectively precludes photobleaching, and excitation around 975 nm markedly suppresses both scattering and endogenous autofluorescence, thus permitting robust, stable, high-contrast imaging at depths extending to several hundred micrometers. The overarching objective of this thesis is to exploit the unique photophysical properties of UCNPs in combination with two complementary strategies, namely adaptive optics (AO) and computational super-resolution optical fluctuation imaging (SOFI), in order to surmount the inherent trade-off between imaging depth and spatial resolution in nonlinear microscopy.

This thesis is structured around three major innovations. The first is UCNP-assisted direct AO microscopy. We have developed a real-time AO nonlinear laser-scanning microscope that incorporates a Shack-Hartmann wavefront sensor and a deformable mirror. The nonlinear fluorescence from UCNPs sample serves simultaneously as an imaging signal and as intrinsic guide stars for direct wavefront sensing. By applying real-time closed-loop correction, distorted excitation light can be precisely refocused, restoring near diffraction-limited spots even in large-aberrations sample. This correction results in significant improvements in image sharpness and signal strength under CW excitation.

The second innovation is homologous dual-emission upconversion AO microscopy (HDU-AOM). This approach employs $\text{Tm}^{3+}/\text{Yb}^{3+}$ co-doped UCNPs' dual 455 nm/800 nm emission: the 800 nm emission for aberration measurement (guide-star) in deep tissues and the 455 nm emission for high-resolution imaging at matching depths. Using a home-built nonlinear laser scanning microscope with a 975 nm CW laser, we achieved near-diffraction-limited imaging (~ 480 nm laterally) at a $500\text{ }\mu\text{m}$ depth in the mouse brain environment with significant optical aberrations, thereby directly coupling near-infrared sensing with visible-band imaging to effectively overcome the depth-resolution trade-off.

The third innovation is speckle-based upconversion nonlinear SOFI (SUN-SOFI). In this approach, we replace molecular blinking with dynamically modulated speckle illumination generated by a spatial light modulator. Speckle-induced intensity variations supply the fluctuation statistics necessary for cumulant analysis while preserving the nonlinear excitation and high-photostability benefits of UCNPs. These fluctuations are subsequently analyzed using high-order SOFI cumulants to recover spatial information beyond the diffraction limit. Experimental results demonstrate approximately a twofold resolution enhancement over traditional wide-field imaging, achieving lateral resolution down to ~ 160 nm with sixth-order analysis. The method is straightforward to implement and preserves the low phototoxicity and high-resolution of NIR-driven UCNPs excitation, making it particularly attractive for live-cell super-resolution applications.

Collectively, by integrating UCNPs with AO and computational super-resolution imaging, this thesis demonstrates high-resolution deep-tissue and super-resolution imaging under CW excitation with minimal photodamage. Leveraging the superior optical properties of UCNPs, including high-order nonlinearity, dual-emission capability, extended penetration depth, and exceptional photostability, enables sub-micron spatial resolution at the single-nanoparticle level within scattering tissues, thereby strengthening the capacity of nonlinear microscopy for real-time monitoring of

deep cellular dynamics. These advances significantly extend the potential of nonlinear optical microscopy for biomedical research and clinical translation.

PUBLICATIONS ARISING FROM THE THESIS

Peer-reviewed Article (# corresponding author)

- 1 **J. Yao**, Z. Yu, B. Wang, Y. Gao, Y. Cai, W. Zheng, Q. Zhan#, P. Lai#, " Speckle-based nonlinear upconversion super-resolution optical fluctuation imaging." (*under review*).
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- 3 **J. Yao**, Y. Gao, Y. Yin, P. Lai, S. Ye#, W. Zheng#, "Exploiting the potential of commercial objectives to extend the field of view of two-photon microscopy by adaptive optics". *Optics Letters* 47, 989-992 (2022).
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- 5 Z. Yu, X. Gao, **J. Yao**, H. Li, Y. Shi, B. Li, Z. Xie, X. Yuan#, P. Lai#, Q. Song#, "Spin-orbit-locking vectorial metasurface holography". *Advanced Materials* 2024, 2415142 (2024).
- 6 Z. Yu, X. Gao, **J. Yao**, Z. Wang, T. Zhong, Y. Shi, B. Li, P. Lai#, X. Li#, Q. Song#, "A spatial-frequency patching metasurface enabling super-capacity perfect vector vortex beams". *eLight* 4, 21(2024).
- 7 Z. Yu, H. Li, W. Zhao, P-S. Huang, Y-T Lin, **J. Yao**, W. Li, Q. Zhao, P. H. Wu, B. Li, P. Genevet, Q. Song# and P. Lai#, "High-security learning-based optical encryption assisted by disordered metasurface". *Nature Communications* 15, 2607 (2024).
- 8 C. Li, **J. Yao**, Y. Gao, P. Lai, Y. He#, S. Qi#, W. Zheng#, "Extending field-of-view of two-photon microscopy using deep learning", *Chinese Journal of Lasers* 50, 9 (2023).

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- 10 Y. He, **J. Yao**, L. Liu, Y. Gao, J. Yu, S. Ye, H. Li, W. Zheng#. "Self-supervised deep-learning two-photon microscopy". *Photonics Research* 11, 1 (2022).

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LIST OF ABBREVIATIONS

Abbreviation	Full name
AO	adaptive optics
CW	continuous-wave
CR	cross-relaxation
DM	deformable mirror
ESA	excited-state absorption
ExM	expansion microscopy
FOV	field of view
FED	fluorescence emission difference
FRAP	fluorescence recovery after photobleaching
FRET	förster resonance energy transfer
FE-UPCON	frequency-encoded upconversion microscopy
GFP	green fluorescent protein
GS	guide-star
HDU-AO	homologous dual-emission upconversion adaptive
LFM	light-field microscopy
LRET	luminescence resonance energy transfer
MINFLUX	minimal fluorescence photon flux microscopy
MPM	multiphoton microscopy
NIR	near-infrared

NIRES	near-infrared emission saturation
NLSM	nonlinear laser scanning microscopy
NLOM	nonlinear optical microscopy
NA	numerical aperture
OL	objective lens
ONE	one-step nanoscale expansion
OCT	optical coherence tomography
PPA	parallel photon avalanche
PA	photoacoustic
PALM	photoactivation localization microscopy
PMT	photomultiplier tube
PMT	photomultiplier tube
PA	photon avalanche
PSF	point spread function
PAINT	points accumulation for imaging in nanoscale topography
PDMS	polydimethylsiloxane
RIM	random illumination microscopy
RESOLFT	reversible saturable optical fluorescence transitions microscopy
SHG	second-harmonic generation
SHWS	Shack-Hartmann wavefront sensor

SNR	signal-to-noise ratio
SLM	spatial light modulator
SUN	speckle-based upconversion nonlinear
STED	stimulated emission depletion microscopy
STORM	stochastic optical reconstruction microscopy
SIM	structured illumination microscopy
SEE	super-linear excitation-emission
SL-ISM	super-linear image scanning microscopy
SOFI	super-resolution optical fluctuation imaging
SMED	surface migration emission depletion
3PA	three-photon absorption
TEM	transmission electron microscopy
2PA	two-photon absorption
UCL	upconversion luminescence
UCNP	upconversion nanoparticle
VIS	visible

1 INTRODUCTION

1.1 Overview of Fluorescence Microscopy

1.1.1 Principle of Fluorescence

Fluorescence is a photophysical process involving molecules known as fluorophores [1]. As illustrated in Fig. 1-1, a fluorophore absorbs a photon of higher energy and is promoted to its first excited singlet state S_1 . It then relaxes rapidly via nonradiative processes to the lowest vibrational level within the S_1 state. Finally, the molecule returns to the ground electronic state (S_0) by emitting a photon with lower energy. The difference between absorption and emission wavelengths, known as the Stokes shift, results from vibrational relaxation and solvent reorientation in the excited state and enables spectral separation of excitation and emission bands.

The use of fluorescence as an analytical tool began to flourish in the nineteenth century with Sir George Stokes's pioneering work on photoluminescence [2]. Stokes demonstrated that certain substances emit light at longer wavelengths than those they absorbed and coined the term "fluorescence". In the early twentieth century, advances in spectroscopic instrumentation allowed precise measurement of excitation and emission spectra and quantitative determination of quantum yields. Chemists synthesized the first high-efficiency organic fluorophores such as fluorescein [3-5] and rhodamine[6], whose bright, narrow emission bands and tunable spectral properties enabled rapid growth of fluorescence spectroscopy across chemistry and biology. Later, the discovery and cloning of green fluorescent protein (GFP) [7] from jellyfish introduced a genetically encoded fluorophore that could be fused to native proteins, revolutionizing live-cell imaging and biosensing. More recently, inorganic nanocrystals known as quantum dots [8] and novel near-infrared dyes [9,10] have extended the accessible spectral range, improved photostability, and enabled multiplexed detection, laying the groundwork for modern applications in single-molecule tracking, super-

resolution imaging, and in-vivo diagnostics.

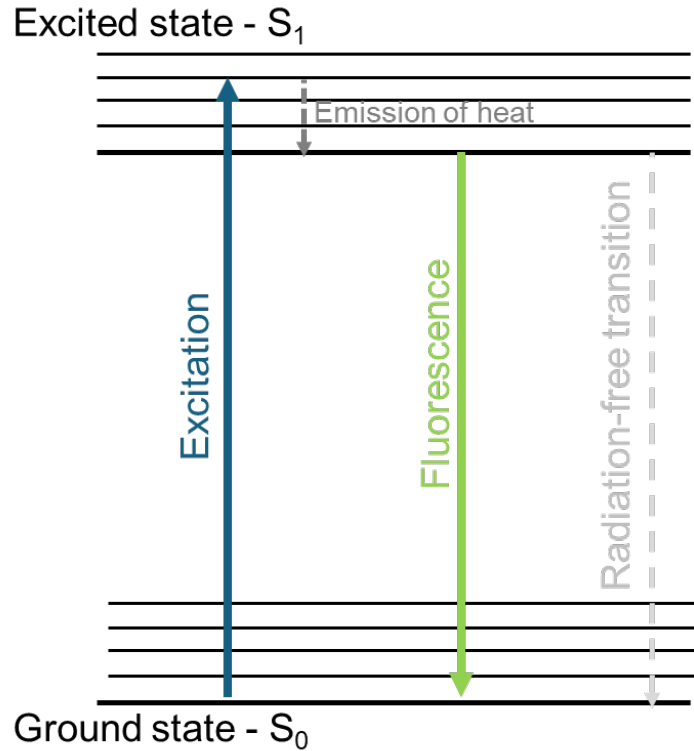


Fig. 1-1 Simplified Jablonski diagram for fluorescence excitation and emission. Vertical lines represent transitions between the ground electronic manifold (S_0) and an excited manifold (S_1). The blue arrow indicates photon absorption (excitation), the green arrow depicts radiative decay (fluorescence), and the dashed gray arrow denotes nonradiative relaxation pathways.

1.1.2 Wide-Field Microscopy

Wide-field fluorescence microscopy [11] uses a conventional inverted or upright light-microscope layout in which excitation and emission light share the same objective. Figure 1-2 shows a standard wide-field microscopy configuration. Excitation light from a broadband source is spectrally confined to the fluorophore's absorption band by an excitation filter. The filtered light is reflected by a dichroic beamsplitter into the objective's back aperture, forming a uniform illumination cone at the specimen. Fluorescence emitted in all directions is collected by the same objective, transmitted back through the dichroic beamsplitter and an emission filter to remove residual excitation light, and then imaged onto a camera or eyepiece.

In this configuration, the excitation filter selects the appropriate excitation wavelengths,

the dichroic beamsplitter directs those wavelengths into the sample while allowing longer-wavelength emission to pass, and the emission filter prevents any remaining excitation light from reaching the detector. Illumination typically combines a halogen lamp for bright-field inspection and a high-pressure mercury arc lamp for fluorescence excitation. The intense spectral lines of the mercury lamp are matched to the fluorophore's absorption peaks by the excitation filter to maximize photon yield, while the emission filter and dichroic beamsplitter efficiently reject unwanted light.

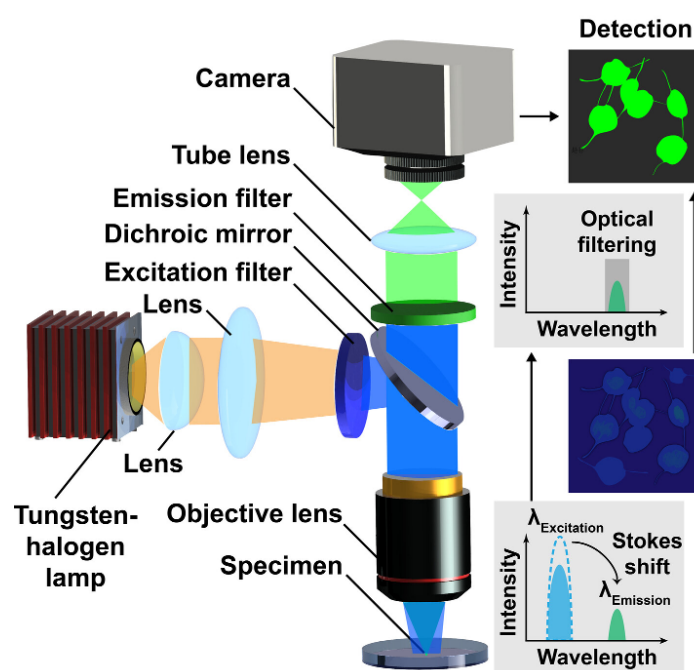


Fig. 1-2 Schematic of a wide-field fluorescence microscope [11]. A tungsten-halogen lamp illuminates the specimen through an excitation filter and dichroic mirror. Fluorescence emitted by the sample is collected by the objective, passes back through the dichroic, and is focused by a tube lens onto a camera after an emission filter. Insets illustrate the Stokes shift between excitation and emission spectra and the spectral selection by the emission filter.

Because the entire field of view is illuminated simultaneously, wide-field microscopy achieves high photon efficiency and frame rates in the order of milliseconds. This makes it ideally suited for observing rapid live-cell dynamics or detecting single molecules. Its simple optical path and compatibility with standard microscope stands have established wide-field fluorescence microscopy as the foundational imaging mode that supports more advanced methods.

Building on this foundational mode, several super-resolution extensions have been developed to surpass the diffraction limit, such as structured illumination microscopy (SIM) [12], photoactivation localization microscopy (PALM) [13], and stochastic optical reconstruction microscopy (STORM) [14]. Wide-field approaches have also been adapted for instantaneous volumetric imaging by encoding depth information in a single snapshot. Light-Field Microscopy (LFM) [15] captures both spatial and angular distributions of light using a microlens array at the image plane, enabling three-dimensional reconstructions of volumes hundreds of micrometers thick from one exposure. Multifocus LFM [16] splits the image onto multiple focal planes on a single sensor to achieve high-speed, large-volume imaging without mechanical scanning. These super-resolution and volumetric variants retain the simplicity and rapid acquisition advantages of wide-field microscopy while significantly extending their ability to resolve more structural details.

1.1.3 Confocal Microscopy

Confocal microscopy was first demonstrated by Marvin Minsky in the late nineteen fifties to achieve optical sectioning and reject out of focus light [17,18]. In its simplest form, a diffraction-limited laser beam is focused through a high numerical aperture (NA) objective into a single point within the specimen, as shown in Fig. 1-3a. Fluorescence emitting from that focal volume is collected by the same objective and then passes through a spatial pinhole positioned in a plane conjugated to the focal plane. Only light originating at the focus traverses the pinhole, while fluorescence excited above or below the focal plane is blocked. By scanning the excitation spot across the x and y plane and recording the signal at each position, a high contrast two-dimensional optical section is generated.

Recent advances in scanning mechanisms have greatly increased both speed and sensitivity [19,20]. Galvanometer driven mirrors permit flexible scan patterns at moderate frame rates. Resonant scanners oscillate at kilohertz frequencies to achieve video rate imaging [21]. For very high-speed applications a spinning disk incorporating

an array of pinholes, known as a Nipkow disk, illuminates and reads out thousands of points in parallel [22-24]. These developments enable time-lapse three-dimensional recordings of living specimens with minimal photodamage.

The intrinsic optical sectioning of confocal systems yields axial resolution on the order of five hundred to seven hundred nanometers under high NA conditions, with lateral resolution of approximately two hundred nanometers. Adjusting the pinhole diameter balances resolution, signal strength, and sectioning thickness, allowing optimization for thick tissues or single molecule sensitivity.

Confocal microscopy has become a fundamental tool in cell biology and neuroscience [25-27]. It is routinely employed for three-dimensional reconstruction of cellular structures, colocalization of multiple labels [28], fluorescence recovery after photobleaching (FRAP) [29], and Förster resonance energy transfer (FRET) [30] measurements. Its capability for precise optical sectioning has also been integrated into correlative imaging workflows and adapted for super resolution methods [31], establishing confocal imaging as the workhorse of high contrast three-dimensional fluorescence microscopy.

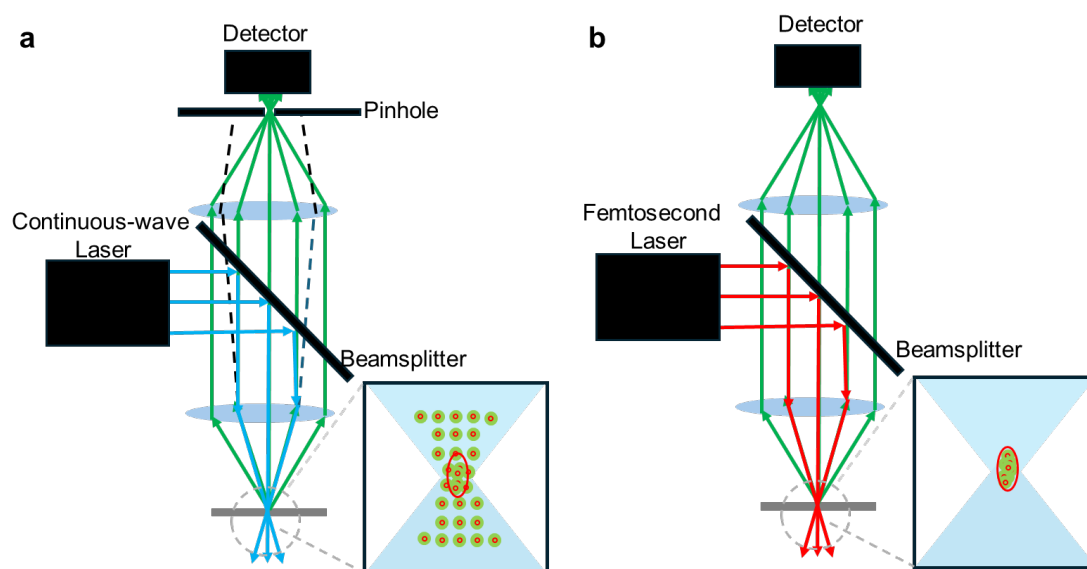


Fig. 1-3 Comparison of confocal and multiphoton laser-scanning microscopy. (a) Confocal microscopy uses CW excitation (blue) and a physical pinhole in the detection path to reject out-of-focus fluorescence (inset). The pinhole improves optical sectioning but attenuates signal from deeper focal

planes. **(b)** Multiphoton microscopy employs femtosecond pulsed excitation (red) at longer wavelengths, which confines fluorescence generation to the focal volume (inset) and obviates the need for a pinhole. Longer excitation wavelengths also enable deeper tissue penetration.

1.1.4 Multiphoton Microscopy

In contrast to confocal microscopy's reliance on a physical pinhole to reject out-of-focus light, multiphoton microscopy (MPM) confines excitation intrinsically through the simultaneous absorption of two or more infrared photons, as shown in Fig. 1-4. The principle was first predicted by Maria Göppert-Mayer in 1931 [32] and experimentally confirmed by Kaiser and Garrett in $\text{CaF}_2:\text{Eu}^{2+}$ in 1961 [33], laying the groundwork for Denk, Strickler and Webb's first two-photon laser-scanning fluorescence microscope in 1990 [34]. By eliminating the pinhole and using nonlinear excitation, true three-dimensional optical sectioning is achieved with dramatically reduced photobleaching and photodamage compared to confocal methods, as illustrated in Fig. 1-3b.

In practice, taking two-photon microscopy as an example, a mode locked titanium sapphire laser delivers femtosecond pulses at approximately 80 MHz repetition rates, with excitation wavelengths in the 700 to 1000 nm range to maximize tissue penetration and minimize scattering [35]. Beam steering is performed by galvanometer mirrors or resonant mirrors that raster the focal spot across the sample, and emitted fluorescence is collected by the same high NA objective and detected by photomultiplier tubes or hybrid detectors. This configuration removes the need for physically descanned light paths and enables efficient signal collection even from deep within scattering specimens.

Because excitation is confined to a sub-femtoliter focal volume, MPM achieves superior signal-to-noise ratio. Reduced scattering of infrared light permits imaging depths of several hundred microns, and three-photon excitation at longer wavelengths (e.g., 1700 nm) has pushed depths beyond one millimeter in intact brain [36]. Second- and third-harmonic generation provides label-free contrast for structures such as collagen and myelin, expanding the modality's versatility.

Recent advances in MPM hardware and computational methods have greatly expanded their performance and accessibility. Ultrafast fiber lasers now deliver high peak power femtosecond pulses over a broadly tunable infrared spectrum in compact cost effective systems making deep tissue imaging more accessible to routine laboratories [37]. Integrated adaptive optics (AO) modules that use deformable mirrors or spatial light modulators correct sample induced aberrations in real-time restoring diffraction limited resolution hundreds of microns below the tissue surface [38]. Techniques such as FACED accelerate volumetric imaging [39], enabling video-rate acquisition across large fields of view. The emergence of multiphoton light sheet microscopy that combines plane illumination with multiphoton excitation has enabled rapid low photodamage imaging in intact embryos and organoids [40,41]. Hybrid two- and three-photon platforms such as DEEPscope [42] capture large-scale neural activity and structural detail at single-cell resolution deep within intact brain.

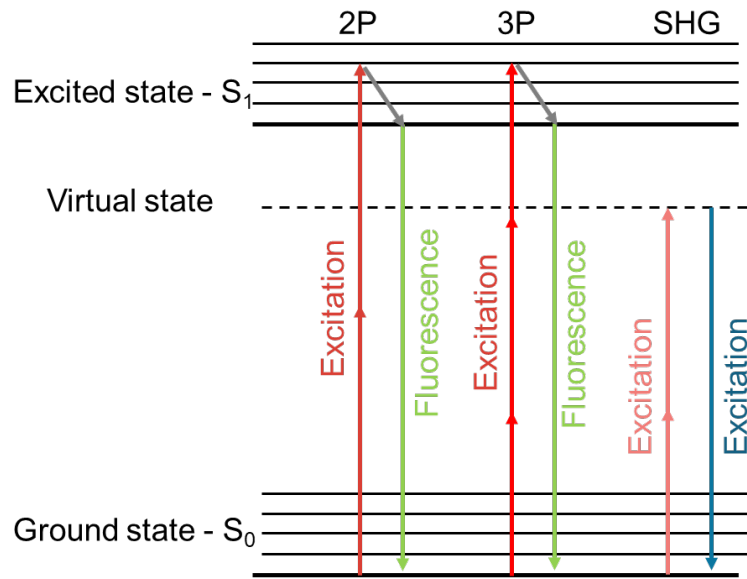


Fig. 1- 4 Energy-level diagram comparing two-photon absorption (2PA), three-photon absorption (3PA), and second-harmonic generation (SHG) processes. Red arrows indicate nonlinear excitation pathways via simultaneous absorption of two or three infrared photons from the ground state S_0 to the real excited state S_1 via virtual intermediate levels. Green arrows denote fluorescence emission from S_1 back to S_0 . In SHG, the pink arrow shows absorption of a single fundamental photon into a virtual state and the blue arrow represents coherent emission

of a second-harmonic photon without populating S_1 .

1.1.5 Super-resolution Microscopy

In optical microscopy, diffraction imposes a fundamental limit on spatial resolution. As Ernst Abbe first described in 1873 [43], two objects cannot be distinguished if they are closer than approximately half the wavelength of light used. In the latter two decades, a series of emerging super-resolution microscopy techniques emerged one after another, successfully breaking this limit that was once considered insurmountable.

One class of methods relies on tailoring the illumination field. Stimulated emission depletion microscopy (STED) [44,45] uses a focused excitation beam overlapped with a doughnut-shaped depletion beam to suppress fluorescence in the periphery of the focus, confining emission to a region whose size shrinks as depletion intensity increases. The same concept is generalized in reversible saturable optical fluorescence transitions microscopy (RESOLFT) [46] with photoswitchable proteins. SIM [47,48] uses patterned excitation to create moiré fringes that encode sub-diffraction details into the recorded image; Random illumination microscopy (RIM) adopts same speckle illumination concept and blind deconvolution. Recently, minimal fluorescence photon flux microscopy (MINFLUX) [49] and MINSTED [50] exploit a movable zero of intensity to localize single molecules with nanometer precision at minimal photon dose, setting a new benchmark for optical nanometrology.

A second major category is localization-based single-molecule microscopy [51]. In these techniques individual fluorophores are stochastically activated or blink in sparse subsets, allowing their point spread functions (PSFs) to be fitted to determine molecular positions with precision of tens of nanometers or better. PALM [52] and STORM [14] achieve roughly 10 nm precision using photoswitchable proteins or organic dyes. DNA-based points accumulation for imaging in nanoscale topography (PAINT) [53] and related PAINT schemes employ transient DNA hybridization to circumvent photobleaching and enable multiplexing. Fluctuation-analysis approaches such as SOFI [54] compute higher-order correlations to gain resolution without isolating single

emitters.

Certain artifice super-resolution techniques are designed for special applications or imaging tools. 4-Pi microscopy narrows the axial PSF with two coherently aligned objectives, delivering nearly isotropic resolution around one hundred nanometers. Expansion microscopy (ExM) [55] embeds fixed specimens in a swellable hydrogel, enlarging four- to ten-fold structures. Image scanning microscopy (ISM) [56] swaps the confocal pinhole for a detector array and, through pixel reassignment followed by deconvolution, raises lateral resolution by roughly $\sqrt{2}$ without sacrificing optical sectioning or photon efficiency.

In more recent years, deep learning has become a powerful tool to further driver the development of super-resolution imaging. Convolutional neural networks can map low-resolution or noisy inputs to high-resolution outputs taking advantage of a priori knowledge, cutting acquisition time and excitation dose [57-59]. Dedicated networks for SIM [60], STED [59], SMLM [61] and SOFI [62] remove artefacts and denoise in real time, enabling video-rate reconstruction. With growing public datasets and self-supervised paradigms, learning-based methods are rapidly complementing hardware solutions and opening avenues for long-term, multi-scale, and low-phototoxic live imaging.

1.2 Adaptive Optics

1.2.1 Introduction of Adaptive Optics

Biological specimens and microscope optical setup introduce a variety of wavefront aberrations that degrade image quality, as shown in Fig. 1-5. Common examples include spherical aberration, which arises from refractive-index mismatch and leads to a broadened PSF; coma, which skews the focal spot off axis; astigmatism, which produces different focal planes along orthogonal meridians; and higher-order distortions that further blur fine features. These aberrations increase background noise, reduce contrast, and limit achievable imaging depth, especially in thick or

inhomogeneous tissues.

AO offers a practical solution by actively measuring and correcting these wavefront errors [38,63,64]. First, the distorted wavefront is measured or calculated, directly or indirectly. Next, a control algorithm computes the complementary phase map to a programmable optical element, which is then used to apply the map and restore a diffraction-limited focus. By closing the loop of measuring and correcting aberrations, AO compensates both static and dynamic aberrations caused by system distortions or sample scattering, leading to sharper images, higher signal throughput, and deeper penetration into scattering media. These together improve the performance of fluorescence microscopy techniques.

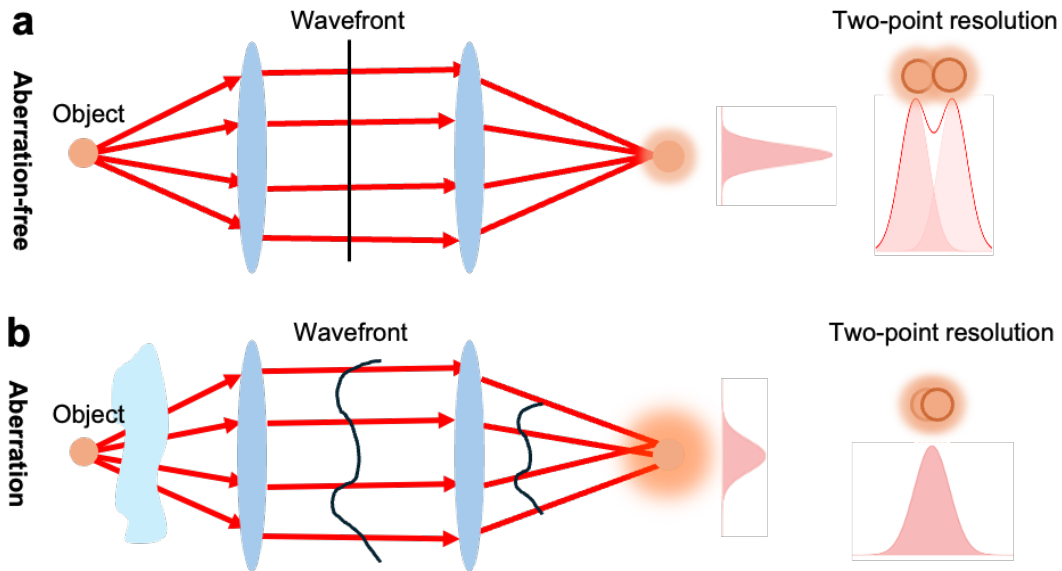


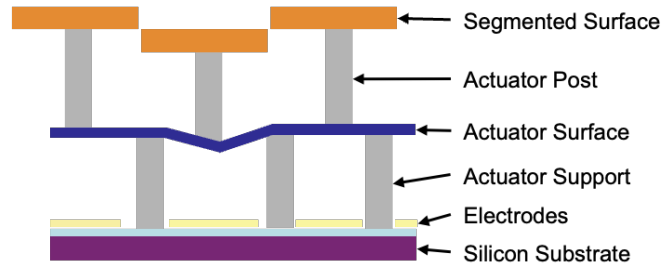
Fig. 1-5 Effect of wavefront aberrations on focus and resolution. (a) In the aberration-free case, planar wavefronts produce a diffraction-limited focus (center) and enable two adjacent point sources to be resolved (right). **(b)** When the wavefront is distorted (wavy lines), the focus broadens and peak intensity decreases (center), and two closely spaced points merge into an unresolved blur (right).

1.2.2 Corrective Elements

Deformable mirrors. Deformable mirrors (DMs) sculpt optical wavefronts by mechanically deforming a reflective surface via an underlying actuator array. In the segmented architecture, each mirror pixel is an independent facet mounted on its own

actuator post and driven by a piezoelectric element. The Boston Micromachines DM140A-35-P01 exemplifies this design, providing several-micrometer strokes and kilohertz-class response times across its array of hexagonal segments, as shown in Fig. 1-6a. This arrangement excels at correcting low to mid-order aberrations with high dynamic range and rapid update rates. By contrast, the continuous-face-sheet architecture uses a single uninterrupted membrane supported by a dense grid of microelectromechanical actuators, as in the ALPAO DM97-15, as shown in Fig. 1-6b. Hundreds of closely spaced posts move by sub-micrometer amounts to deform the membrane smoothly, enabling precise compensation of complex higher-order distortions. Although individual actuator strokes are smaller than those of bulk piezo devices, the fine actuator pitch and high fill factor yield exceptional spatial control in real time, making MEMS DMs ideal for wavefront flattening over broad spectral bands. When placed in a pupil-conjugate plane, both segmented and continuous DMs restore planarity to distorted wavefronts, recover diffraction-limited focus, and enable advanced imaging and beam-shaping applications in astronomy and microscopy. DMs employ piezoelectric or MEMS actuator arrays in a conjugate pupil plane to deliver large-stroke, fast (kilohertz-scale) correction of low- and mid-order aberrations. However, their relatively coarse actuator counts limits spatial resolution.

a Segmented Type



b Continuous Type

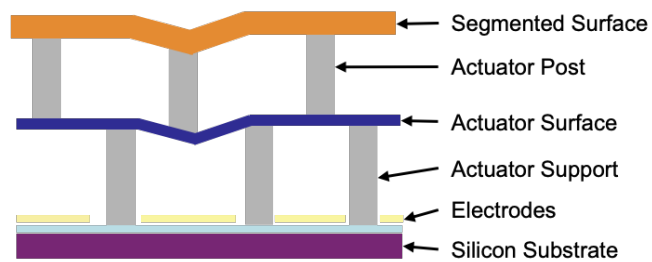


Fig. 1-6 Segmented vs. continuous deformable mirror architectures. (a) Segmented Type: An array of individually controlled mirror segments, each supported by its own actuator post and electrode, allowing piecewise adjustment of the optical wavefront. Example: Boston Micromachines Corp. DM140A-35-P01. **(b) Continuous Type:** A single continuous reflective membrane deformed by multiple underlying actuator posts and a common support layer, enabling smooth, global wavefront shaping. Example: ALPAO DM97-15.

Spatial light modulators. By contrast, spatial light modulators (SLMs) are pixelated liquid-crystal devices with tens of thousands (e.g., 1920×1080) of elements. Applying an electric field tunes the refractive index of each element and thus varies optical path length. Figure 1-7a shows a reflective liquid-crystal SLM module and 7b illustrates a simplified cross section of one pixel: at zero field the molecules lie parallel to the substrate and impart negligible phase delay; at half the maximum field they tilt to introduce a π phase shift; at full field they stand nearly upright to impart a 2π retardation to the reflected wavefront. Although SLM update rates of tens to hundreds of hertz are slower than the kilohertz rate of DMs, their very high pixel density enables precise correction of complex high-order aberrations and the programmable generation of engineered beams such as Bessel beams or multifocal arrays.

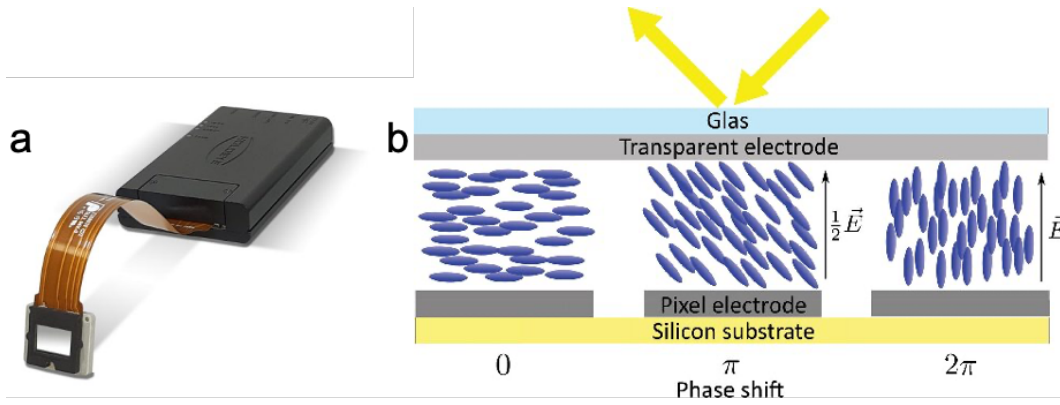


Fig. 1-7 Liquid-crystal spatial light modulator (SLM) principle. (a) Photograph of a reflective liquid-crystal SLM module. (b) Simplified cross-section of one pixel showing how the applied electric field E across the transparent top electrode and the pixel electrode reorients the liquid-crystal molecules. At zero field, the molecules lie mostly parallel to the substrate, yielding negligible phase delay. At intermediate field ($E/2$), they tilt to produce a π phase shift. At full field (E), they stand nearly upright, imparting a 2π retardation to the reflected wavefront.

Thus, DMs excel at rapid, large-stroke correction of low to mid order aberrations while SLMs offer fine-resolution high-order correction and advanced beam shaping. Together they form a powerful toolkit for wavefront control in microscope imaging.

1.2.3 Indirect AO

Indirect AO, also called sensorless or image based AO, infers system- and sample-induced aberrations by optimizing an image quality metric rather than by measuring the wavefront directly [65], as shown in Fig. 1-8. This approach works well in systems where inserting a guide star is impractical or where the fluorescence signal is too weak for a conventional wavefront sensor. The general workflow has three steps. First, a trial phase pattern is applied to the corrective element (SLM/DM). Second, an image is acquired and a predefined metric, such as brightness, sharpness or contrast, is computed. Third, the dependence of that metric on the applied phase is analyzed to determine the optimal correction map [66].

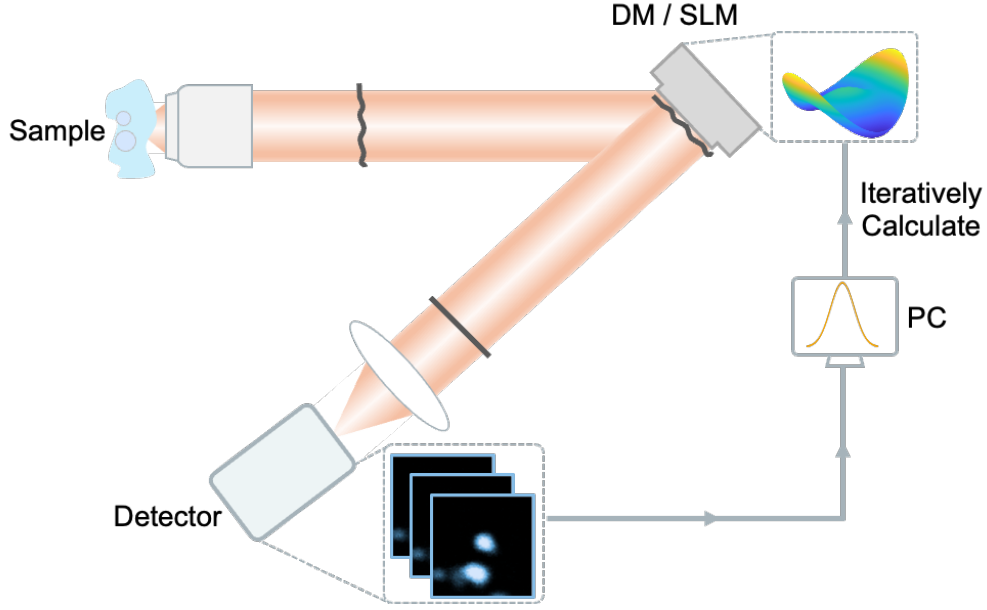


Fig. 1-8 Indirect adaptive-optics scheme based on image feedback. A phase modulator (DM/SLM) is inserted into the excitation path. Scattered light or fluorescence from the sample is collected by the detector to form an image (inset). An optimization algorithm on the PC iteratively adjusts the phase pattern on the DM/SLM to maximize a chosen image metric, such as focal intensity or sharpness, without direct wavefront measurement.

A common implementation uses a modal basis of Zernike polynomials [67], as detailed in Table 1-1 and visualized in Fig. 1-9. Zernike polynomials form a complete set of orthonormal functions defined on the unit circle. In single-index notation the k th polynomial $Z_k(\rho, \theta)$ corresponds to classical aberrations such as astigmatism, coma and spherical aberration. Any aberrated wavefront $\phi(\rho, \theta)$ can be expanded as

$$\phi(\rho, \theta) = \sum_{k=0}^{\infty} C_k Z_k(\rho, \theta), \quad (1-1)$$

where the coefficient C_k quantifies the strength of each mode[68]. Higher order aberrations that contain finer spatial variations require Zernike modes of correspondingly higher index. In high NA microscopy the large range of spatial frequencies in the pupil function makes Zernike decomposition especially powerful for capturing both low and high order distortions.

Index	m	n	Zernike mode	Name
1	0	0	1	Position
2	1	1	$2r\cos\theta$	Tip
3	1	-1	$2r\sin\theta$	Tilt
4	2	0	$\sqrt{3}(2r^2 - 1)$	Defocus
5	2	2	$\sqrt{6}r^2\cos 2\theta$	Astigmatism, 1st order
6	2	-2	$\sqrt{6}r^2\sin 2\theta$	Astigmatism, 1st order
7	3	3	$2\sqrt{2}r^3\cos 3\theta$	Trefoil
8	3	1	$2\sqrt{2}(3r^2 - 2r)\cos\theta$	Coma
9	3	-1	$2\sqrt{2}(3r^2 - 2r)\sin\theta$	Coma
10	3	-3	$2\sqrt{2}r^3\sin 3\theta$	Trefoil
11	4	0	$\sqrt{10}r^4\cos 4\theta$	Quadrafoil
12	4	4	$\sqrt{10}(4r^4 - 3r^2)\cos 2\theta$	Astigmatism, 2nd order
13	4	2	$\sqrt{5}(6r^4 - 6r^2 + 1)$	Spherical aberration
14	4	-2	$\sqrt{10}(4r^4 - 3r^2)\sin 2\theta$	Astigmatism, 2nd order
15	4	-4	$\sqrt{10}r^4\sin 4\theta$	Quadrafoil

Table 1-1 First 15 Zernike polynomials in Noll indexing.

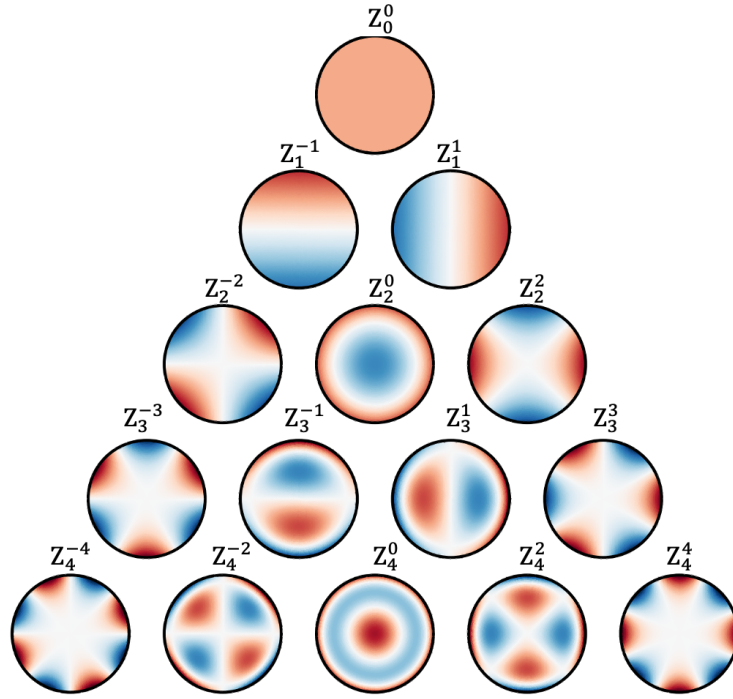


Fig. 1-9 Visualization of the first fifteen Zernike modes.

To implement sensorless correction, each Zernike mode is applied in turn at several amplitudes on a DM/SLM. The image metric is measured for each amplitude and a curve is fitted to identify the optimal amplitude for that mode. Summing the optimal amplitudes for the first few low order modes removes the bulk of the aberration; including higher order modes in subsequent rounds refines the correction and recovers additional resolution.

Alternatively, indirect AO may employ stochastic optimization methods such as stochastic parallel gradient descent or genetic algorithms [69,70]. These methods introduce small random phase perturbations and estimate the gradient of the image metric with respect to the applied phase. Iterating this process converges on the phase map that delivers the best image quality. Although these methods typically require more image acquisitions and therefore operate more slowly than sensor-based AO, they can correct complex high order aberrations without any prior knowledge of the sample optical properties.

Recently, machine learning approaches have accelerated sensorless AO [71]. Deep reinforcement learning and physics-based neural networks have been embedded in

control loops to infer aberrations directly from images, achieving near real-time correction and broad applicability across modalities [72]. Novel techniques such as intensity AO [73] introduce dual-feedback loops to address both intensity inhomogeneity and phase aberrations, expanding the sensorless AO toolkit.

Indirect AO has been successfully applied in various modalities, including SIM [74], optical coherence tomography (OCT) [75], single-molecule localization microscopy [76], and other microscopes' configurations such as confocal [77,78], two-photon [79], light-sheet [80] and super-resolution microscopy [81]. It enables diffraction limited performance in deep tissue imaging where scattering prevents the use of a wavefront sensor and in setups that must avoid additional hardware complexity. The main trade-off lies between correction speed and the level of aberration compensation; the choice of image metric, modal basis and optimization algorithm must match the specimen dynamics and the imaging modality.

1.2.4 Direct AO

Direct wavefront sensing in microscopy employs a dedicated sensor to acquire a distorted optical wavefront and applies its conjugate phase to a DM/SLM in a closed loop configuration to restore diffraction limited resolution deep within scattering specimens [82]. As shown in Fig. 1-10, the most common implementation uses a Shack-Hartmann wavefront sensor (SHWS). In this setup, a microlens array is placed in a plane conjugate to the pupil. Light emitted or back-scattered from the specimen passes through the array and forms a grid of spots on a camera. Each lenslet focuses a sub-aperture of the beam onto the detector; the lateral displacement of each spot relative to a reference grid corresponds to the local wavefront slope. A reconstruction algorithm integrates all measured slopes to recover the full phase error, and the resulting phase map is inverted and applied to the deformable mirror or spatial light modulator. By repeating measurement and correction at rates of several kilohertz, both static aberrations from refractive-index mismatch and dynamic aberrations from sample motion or thermal drift are compensated.

The principle of the SHWS is illustrated in Fig. 1-11. For an aberration-free (planar) wavefront, parallel rays entering each microlens focus to spots aligned on the reference grid at the sensor plane (Fig. 1-11a). When the incoming wavefront is distorted, each microlens receives tilted rays so that its focal spot shifts laterally (red arrows in Fig. 1-11b). Mapping these spot displacements directly encodes the local wavefront slopes, which the reconstruction algorithm then integrates to yield the full phase error.

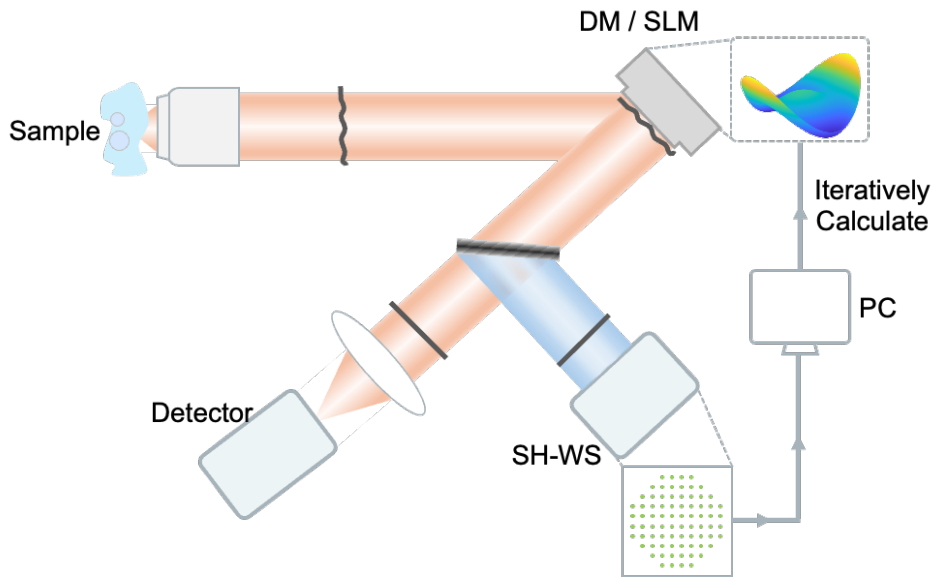


Fig. 1-10 Direct adaptive-optics setup in laser-scanning microscopy. Excitation light (orange) is sent through a phase modulator (DM/SLM) and focused into the sample. Emission or back-scattered light is descanned and split: one path returns to the imaging detector, while the other is diverted to a SHWS (blue). The SHWS measures the distorted wavefront (inset spot array), and a reconstruction algorithm on the PC computes the required corrective phase map. This map is immediately loaded onto the DM/SLM for real-time aberration compensation.

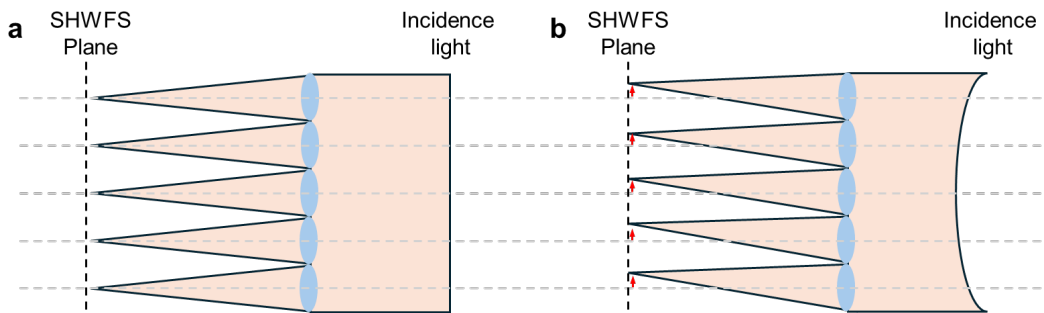


Fig. 1-11 The principle of a SHWS. (a) For a planar (aberration-free) wavefront, parallel rays

across each sub-aperture are refocused by the microlens array to form spots aligned on the reference grid at the SHWS plane (dashed vertical line). **(b)** When the incoming wavefront is distorted, each microlens receives tilted rays, so its focal spot is displaced laterally (red arrows) relative to the reference positions. The map of these spot displacements directly encodes the local wavefront slopes.

Direct wavefront sensing requires a sufficiently bright guide signal. In microscopy this guide often takes the form of a fluorescent bead or a nonlinear contrast source such as second-harmonic-generation labels. Backscattered light can also serve as a guide. When such a signal is available, direct sensing provides the fastest route to restoring diffraction limited performance deep in scattering tissue and in thick specimens.

Alternative direct sensors broaden the AO toolkit beyond SHWS. The curvature sensor first introduced by Roddier [83] in atmospheric astronomy acquires two defocused intensity images on either side of the pupil plane and computes their difference to infer local wavefront curvature, which is then converted to a phase map via phase retrieval algorithms. The pyramid wavefront sensor devised by Ragazzoni splits the focus with a four faceted prism into multiple pupil images whose relative intensities encode local wavefront gradients; this design offers adjustable sampling and higher sensitivity to low order errors [84]. In addition, an all-photonics focal-plane wavefront sensor based on a photonic-lantern fiber-mode converter and deep learning has been demonstrated by Norris et al., [85] placing the sensor at the science focal plane to eliminate non-common-path errors and recovering Zernike wavefront errors with high precision from intensity-only measurements. High density lenslet arrays and meta lenslet designs increase slope sensitivity and dynamic range, enabling submicron aberration correction in highly scattering media [86]. Together, these advances cement direct wavefront sensing as the fastest and most reliable route to achieving diffraction limited imaging across two photon confocal and super resolution modalities in challenging biological samples.

1.3 Upconversion-Assisted Nonlinear Microscope Imaging

1.3.1 Upconversion Nanoparticles

Conventional nonlinear fluorescent dyes, such as those used in two-photon microscopy, rely on simultaneous multiphoton absorption via a virtual energy state and require femtosecond pulsed lasers with high peak intensity, often causing photodamage, photobleaching, and strong tissue scattering and autofluorescence background. Upconversion nanoparticles (UCNPs) have emerged as a novel class of nonlinear probes designed to overcome these limitations.

UCNPs are typically inorganic nanocrystals (e.g. NaYF_4) doped with trivalent lanthanide ions, which endow them with unique anti-Stokes emission properties. Under near-infrared (NIR) excitation, these nanoparticles can sequentially absorb two or more low-energy photons and emit a single higher-energy photon in the visible or ultraviolet range. In practice, this means UCNPs can convert deep-penetrating NIR light into shorter-wavelength visible emission, effectively providing access to high-energy (high-resolution) optical signals deep within scattering tissue.

Distinct from traditional two-photon fluorophores, UCNPs achieve upconversion emission through real, stable intermediate energy levels provided by the doped lanthanide ions. A common approach (Fig. 1-12) involves co-doping a sensitizer ion, typically Yb^{3+} , which efficiently absorbs photons around 975 nm, with activator ions like Tm^{3+} or Er^{3+} . Upon NIR excitation, Yb^{3+} ions initially transition to their excited $^2\text{F}_{5/2}$ state and subsequently transfer their absorbed energy stepwise to the activator ions via non-radiative energy transfer processes. The activators, having received this energy, are promoted to higher energy manifolds such as $^3\text{H}_4$, $^3\text{F}_3$, $^1\text{G}_4$, and $^1\text{D}_2$ before ultimately emitting photons at visible wavelengths including 800 nm, 700 nm, 480 nm, and 455 nm. Due to this ladder-like excitation mechanism, UCNPs exhibit much higher energy transfer efficiency compared to simultaneous multiphoton absorption of conventional fluorophores. Consequently, UCNPs can achieve bright emission under relatively low-power CW NIR illumination, significantly simplifying the microscopy configuration

and substantially reducing the phototoxic effects commonly associated with traditional multiphoton imaging methods.

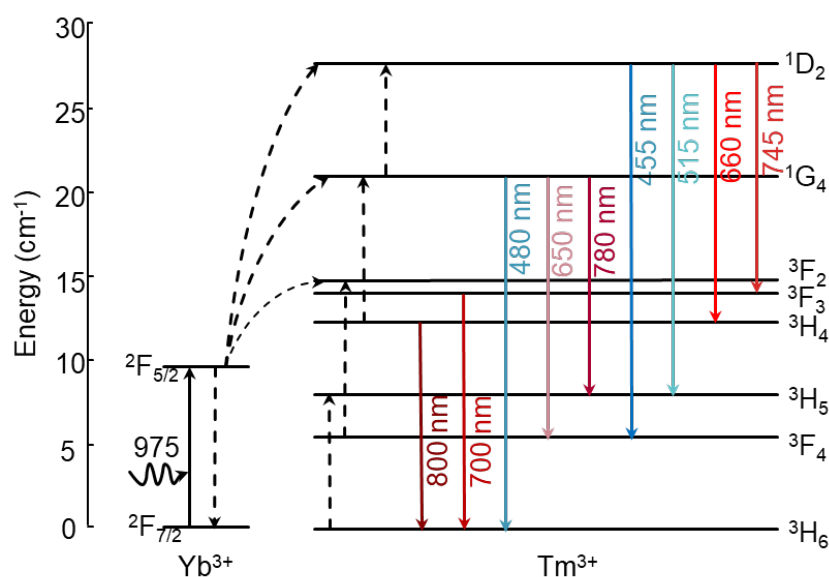


Fig. 1-12 Energy level diagram of Yb³⁺/Tm³⁺ co-doped upconversion nanoparticles. Under 975 nm excitation, Yb³⁺ ions absorb photons and transfer energy to Tm³⁺ ions via non-radiative energy transfer (dashed arrows) and cross-relaxation (CR) processes, sequentially populating the ³H₅, ³H₄, ³F₂, ³F₃, ¹G₄ and ¹D₂ manifolds. Radiative relaxation of Tm³⁺ produces emissions at multiple wavelengths including 800 nm (³H₄→³H₆), 700 nm (³F₃→³H₆), 650 nm (¹G₄→³F₄), 480 nm (¹G₄→³H₆), 455 nm (¹D₂→³F₄), 515 nm (¹D₂→³H₅), 660 nm (¹D₂→³H₄) and 745 nm (¹D₂→³F₃).

Moreover, lanthanide ions exhibit uniquely shielded 4f-4f electronic transitions due to the surrounding inorganic crystal lattice, which endow UCNPs with exceptional photostability. Unlike organic dyes or quantum dots, UCNPs exhibit resistance to photobleaching and do not display blinking behavior, as each nanoparticle contains thousands of collectively emitting dopant ions. This collective emission mechanism results in stable and continuous luminescence output even under prolonged excitation conditions, making UCNPs especially suitable for extended dynamic imaging studies. Additionally, biological tissues possess negligible endogenous upconversion capabilities and exhibit weak absorption of NIR excitation wavelengths, thereby significantly reducing autofluorescence background and minimizing sample heating.

Therefore, UCNPs provide distinct advantages for tissue imaging, particularly for deep-tissue high-resolution imaging:

Deep tissue penetration with low background: NIR excitation light (commonly 800-980 nm) experiences significantly less scattering and absorption in tissue than visible light, allowing UCNP-based microscopy to probe hundreds of microns deep into samples.

Minimal photodamage: Because UCNPs utilize CW NIR excitation, which employs photons of lower energy and avoids the high peak intensities of pulsed lasers, they do not directly excite endogenous biomolecules.

Photostability and no blinking: The inorganic crystalline host and forbidden f-f transitions of lanthanide-doped UCNPs make them highly resistant to photobleaching.

Intrinsic optical sectioning: Upconversion is a nonlinear process, which inherently confines significant emission to the focal volume where the light intensity is highest.

High spatial resolution: The upconversion emission intensity often depends on a high-order nonlinearity, effectively sharpening the point-spread function of the emission and further enhancing lateral resolution and image contrast.

1.3.2 Upconversion Microscopy for Biomedical Imaging

UCNP-based imaging offers several unique advantages for microscopy. Building on these advantages, researchers have demonstrated a variety of innovative biomedical imaging applications using UCNPs. One early breakthrough was the realization of high-contrast *in vivo* upconversion imaging by Nyk, Kumar, Prasad and colleagues [87], who showed that NIR-to-NIR upconversion nanophosphors could enable deep, background-free visualization in live animals. In their study (Fig. 1-13), ~20 nm NaYF₄ nanoparticles co-doped with Yb³⁺/Tm³⁺ were used to emit 800 nm luminescence under 975 nm excitation, thereby operating entirely within the NIR optical window. This approach produced markedly enhanced imaging depth and contrast. In *in vivo* experiment, intravenous injections of the UCNPs into mice allowed the researchers to

capture bright, high-contrast images of internal tissues with essentially zero autofluorescence background. This pioneering work established that upconversion microscopy can overcome the penetration and background limitations of conventional optical imaging, laying the groundwork for subsequent biomedical applications.

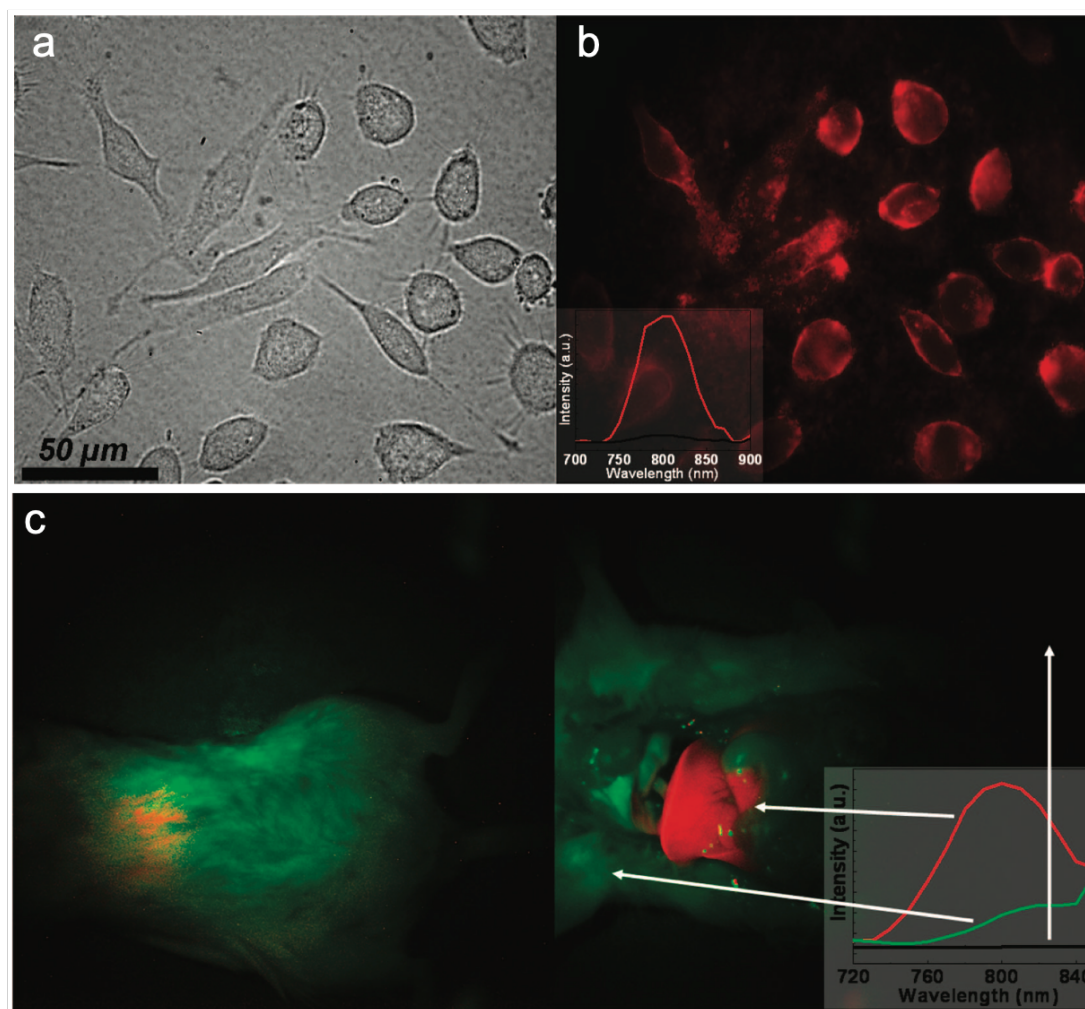


Fig. 1-13 Engineered upconversion nanoparticles for resolving protein interactions inside living cells [87]. (a) Bright-field transmission micrograph of Panc-1 cells after 4 h incubation with NaYF₄: Yb³⁺/Tm³⁺ UCNPs. **(b)** Corresponding upconversion photoluminescence (PL) image recorded under 975 nm CW excitation. **(c)** Multispectral whole-body imaging of a BALB/c mouse 30 min after intravenous injection of the same UCNPs.

Subsequent advances have extended upconversion microscopy to probing biochemical interactions and dynamic processes inside living cells. For example, Drees et al. [88] engineered sub-50 nm UCNPs optimized for enhanced brightness and employed them

as luminescence resonance energy transfer (LRET) donors to monitor protein-protein interactions in live cells. A further milestone in upconversion microscopy was the ability to track single nanoparticles in complex biological environments, enabling the study of fast intracellular transport phenomena. Zeng et al. [89] reported a wide-field upconversion microscopy approach capable of visualizing individual UCNPs moving along neuronal axons with high spatiotemporal resolution.

In parallel, researchers have exploited UCNPs to achieve multiplexed imaging, distinguishing multiple targets in the same sample via upconversion signals. A notable advance by Liisberg et al. [90] introduced frequency-encoded upconversion microscopy (FE-UPCON) as a strategy to separate overlapping UCNP emissions by modulating them at distinct frequencies. Instead of relying on different colors or lifetimes alone, this wide-field approach uses a secondary, periodically modulated excitation source to selectively enhance the luminescence of a specific lanthanide ion in one subset of UCNPs. By applying synchronized dual-wavelength excitation with a chopper wheel, Liisberg and co-workers demonstrated simultaneous imaging of two spectrally similar upconverting nanoparticle species ($\text{Yb}^{3+}/\text{Ho}^{3+}$ versus $\text{Yb}^{3+}/\text{Er}^{3+}$ co-doped) in live cells at video-rate.

Most recently, Haye et al. (2025) [91] reported sub-40 nm molecular upconversion nanoparticles that encapsulate $\sim 1.9 \times 10^3$ Yb/Tb clusters; by combining atomic-level energy architecture with a polymer matrix, these probes maintain high brightness and near-zero autofluorescence in water, enabling long-term live-cell imaging under 980 nm excitation, as shown in Fig. 1-17a. Besides, Kim et al. (2025) [92] demonstrated a nonlinear upconversion microscopy approach using $\text{Yb}^{3+}/\text{Tm}^{3+}$ co-doped NaYF_4 UCNPs under CW excitation, achieving multiphoton-like imaging performance including in vivo deep brain imaging (800 μm depth), wide-field vascular flow imaging, and depth-selective photo-modulation, as shown in Fig. 1-17b.

The continued development of upconversion microscopy points toward broad opportunities in biomedical research and translational medicine. Ongoing efforts to improve UCNP brightness, targeting specificity, and biocompatibility will further

enhance imaging sensitivity and safety, paving the way for potential clinical use in the future.

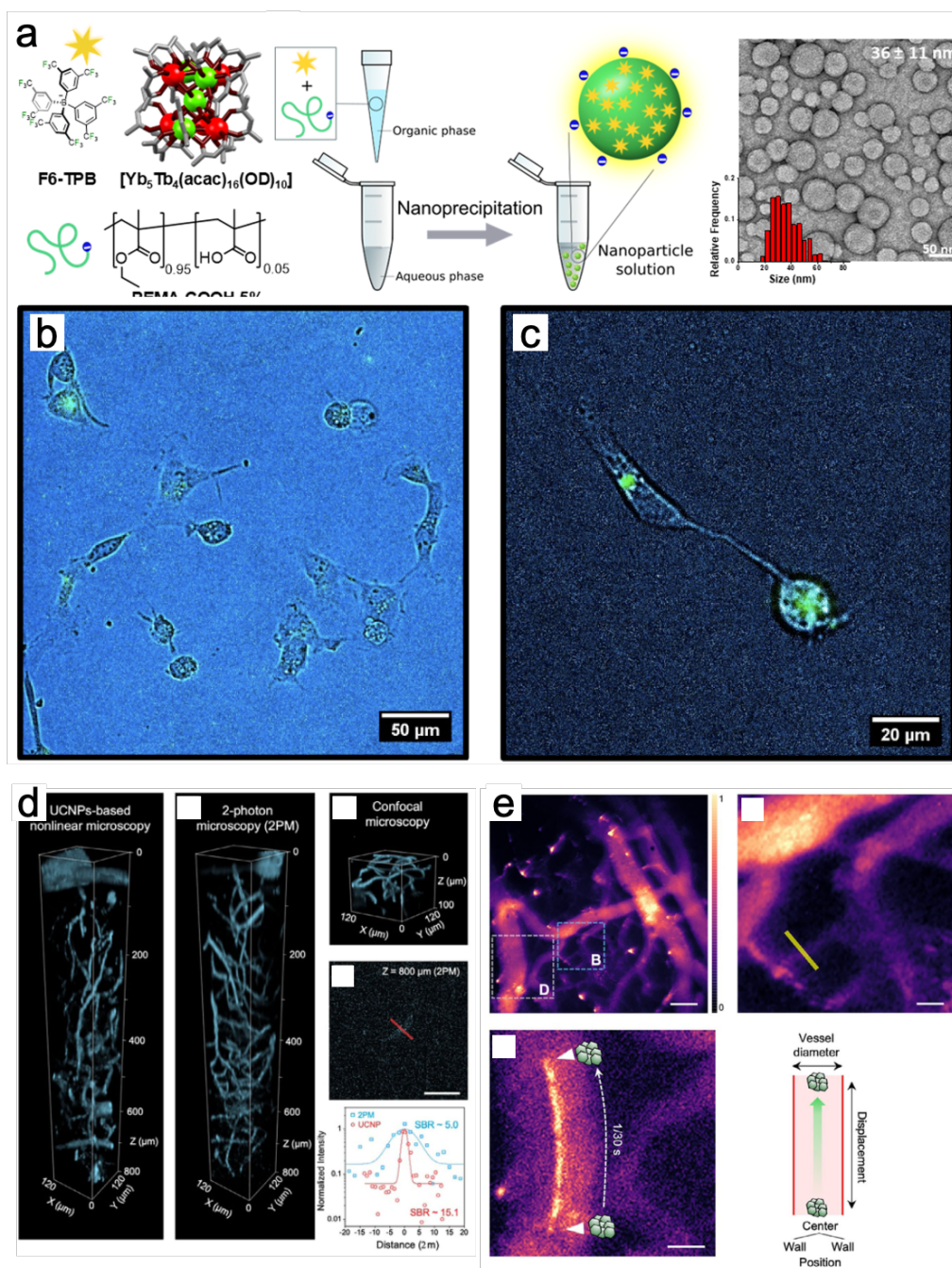


Fig. 1- 14 The most recent progresses of upconversion microscopy for biomedical imaging. (a-c) Molecular Upconversion Nanoparticles for Live-Cell Imaging [91]. (d,e) Unlocking Multimodal Nonlinear Microscopy for Deep-Tissue Imaging under CW Excitation with Tunable Upconverting Nanoparticles [92].

1.3.3 Super-Resolution Microscopy Based on UCNPs

In recent years, UCNPs have emerged as a powerful solution to the key challenges of conventional super-resolution microscopy, notably its independence on high laser power and the resulting phototoxicity. Their combination of NIR excitation, special depletion response, intrinsic multiphoton nonlinearity under CW excitation and exceptional resistance to photobleaching makes this possible. By integrating these features into established super-resolution frameworks, researchers have realized a suite of advanced super-resolution imaging modalities.

Among these techniques, STED microscopy adapted to UCNPs represents a remarkable breakthrough. Conventional STED microscopy typically necessitates high-power pulsed lasers to achieve stimulated emission and depletion of fluorescence at the periphery of the excitation volume. However, UCNP-based systems have revolutionized this paradigm by employing lanthanide ions' unique energy transfer mechanisms, particularly excited-state stimulated emission and interionic cross-relaxation, significantly reducing required depletion intensities. Liu et al. [93] demonstrated that by doping NaYF₄ nanoparticles with high concentrations of Tm³⁺ under 980 nm excitation, an intense cross-relaxation-induced photon-avalanche-like process efficiently established population inversion at the Tm³⁺ intermediate ³H₄ level (Fig. 1-18a). A subsequent low-intensity donut-shaped depletion beam at 808 nm matching the ³H₄ → ³H₆ transition effectively triggered amplified stimulated emission, quenching blue emission and achieving remarkable lateral resolutions down to approximately 28 nm. The required depletion intensities (0.66–9.75 MW/cm²) were far below the gigawatt-level intensities generally required for conventional STED microscopy, alleviating potential photodamage and making it particularly suitable for sensitive biological samples. Simultaneously, Zhan et al. [94] expanded on this concept, demonstrating that interionic cross-relaxation between lanthanide ions could further assist in emission depletion, substantially lowering the laser intensity needed for effective optical modulation (Fig. 1-18b). Utilizing similar lanthanide-doped UCNPs (NaYF₄: Yb³⁺/Tm³⁺), they achieved efficient depletion of upconversion luminescence

through carefully optimized cross-relaxation channels, reaching lateral imaging resolutions around 66 nm using significantly reduced depletion power densities. These complementary studies underline the potential of UCNP-based STED approaches, opening new avenues for super-resolution microscopy with minimal photonic stress, promising significant advances in biomedical imaging, particularly within living or optically dense specimens.

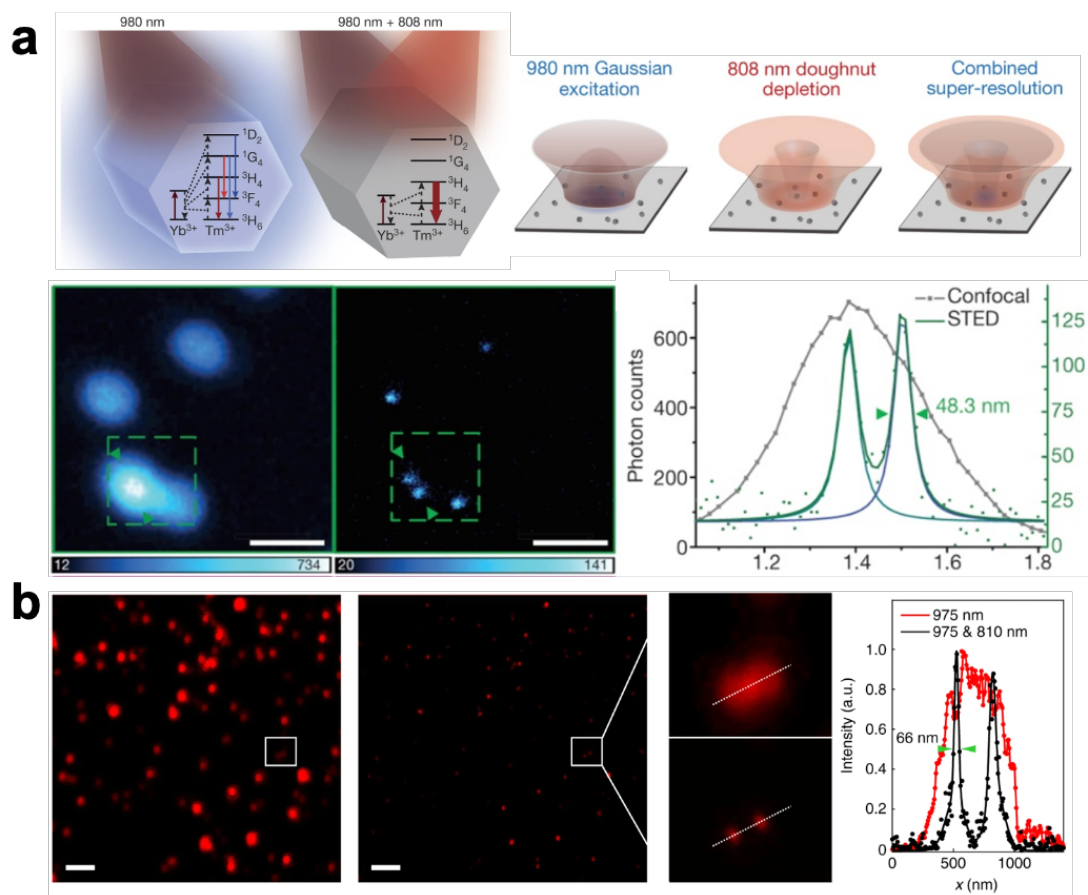


Fig. 1-15 STED microscopy based on UCNPs. (a) Schematic of the super-resolution STED microscopy imaging system based on spatially overlapped Gaussian-shaped excitation and donut-shaped depletion laser beams and lanthanide ion-doped UCNPs with high Tm^{3+} doping and super-resolution STED microscopy results [93]. **(b)** The nanoparticles imaging using high-efficiency emission depletion nanoscopy by employing cross relaxation [94].

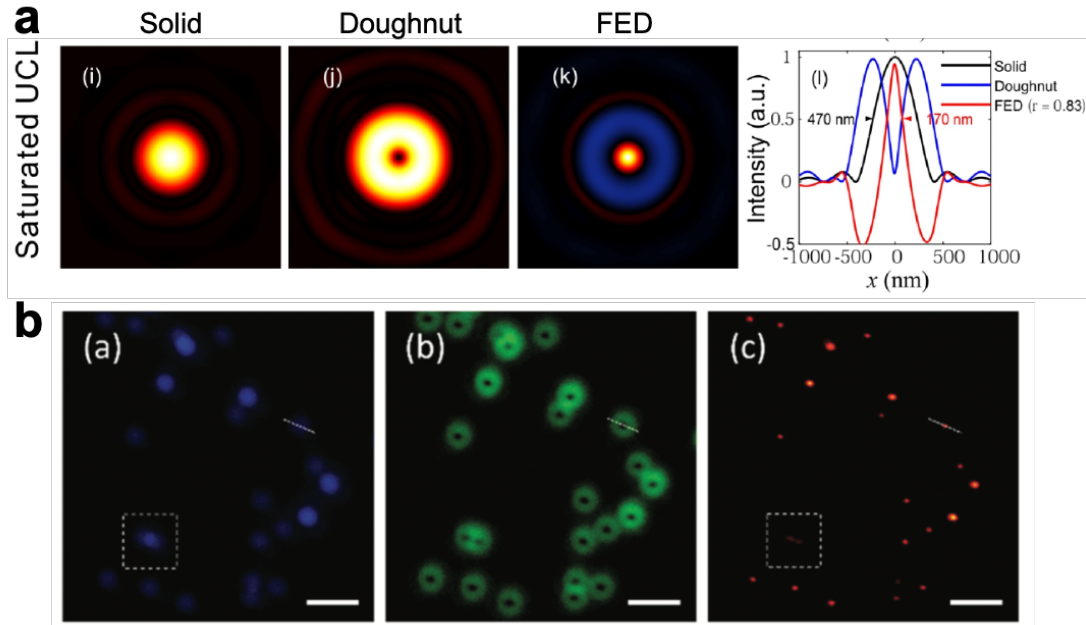


Fig. 1-16 FED microscopy based on UCNPs. (a) the principle of FED microscopy and results [95]. **(b)** One-scan FED nanoscopy imaging of orthogonalized Er@Y@Yb/ Tm nanoparticles [96].

Fluorescence emission difference (FED) microscopy represents another significant advancement among UCNP-based super-resolution imaging modalities, relying on spatial modulation of excitation rather than conventional emission depletion mechanisms. FED microscopy achieves improved resolution by acquiring and subtracting two sequential images, one obtained with a solid Gaussian excitation beam and the other with a hollow, donut-shaped beam. This subtraction method effectively narrows the PSF, significantly enhancing spatial resolution. Importantly, Wu et al. [95] demonstrated that lanthanide-doped UCNPs can substantially boost FED microscopy's performance due to their intrinsic photostability and nonlinear excitation characteristics (Fig. 1-19a). Further refining this method, Huang et al. [96] developed an innovative one-scan FED nanoscopy approach utilizing excitation-orthogonalized UCNPs composed of Er^{3+} -doped cores and $\text{Yb}^{3+}/\text{Tm}^{3+}$ -doped outer shells (Fig. 1-19b). These specially structured UCNPs emitted orthogonally excitable fluorescence at two distinct wavelengths (808 nm and 940 nm), enabling simultaneous dual-channel imaging from one single scanning event.

Another widely adopted method is near-infrared emission saturation (NIRES) nanoscopy [97], which takes advantage of the nonlinear excitation dynamics of UCNPs to create a negative contrast at the periphery of a donut-shaped excitation pattern, which is as shown in Fig. 1-20. Using $\text{Yb}^{3+}/\text{Tm}^{3+}$ -doped UCNPs excited at 980 nm and detecting 800 nm emission, NIRES achieves lateral resolutions as fine as 34 nm under moderate power levels ($\sim 4 \text{ mW}/\text{cm}^2$).

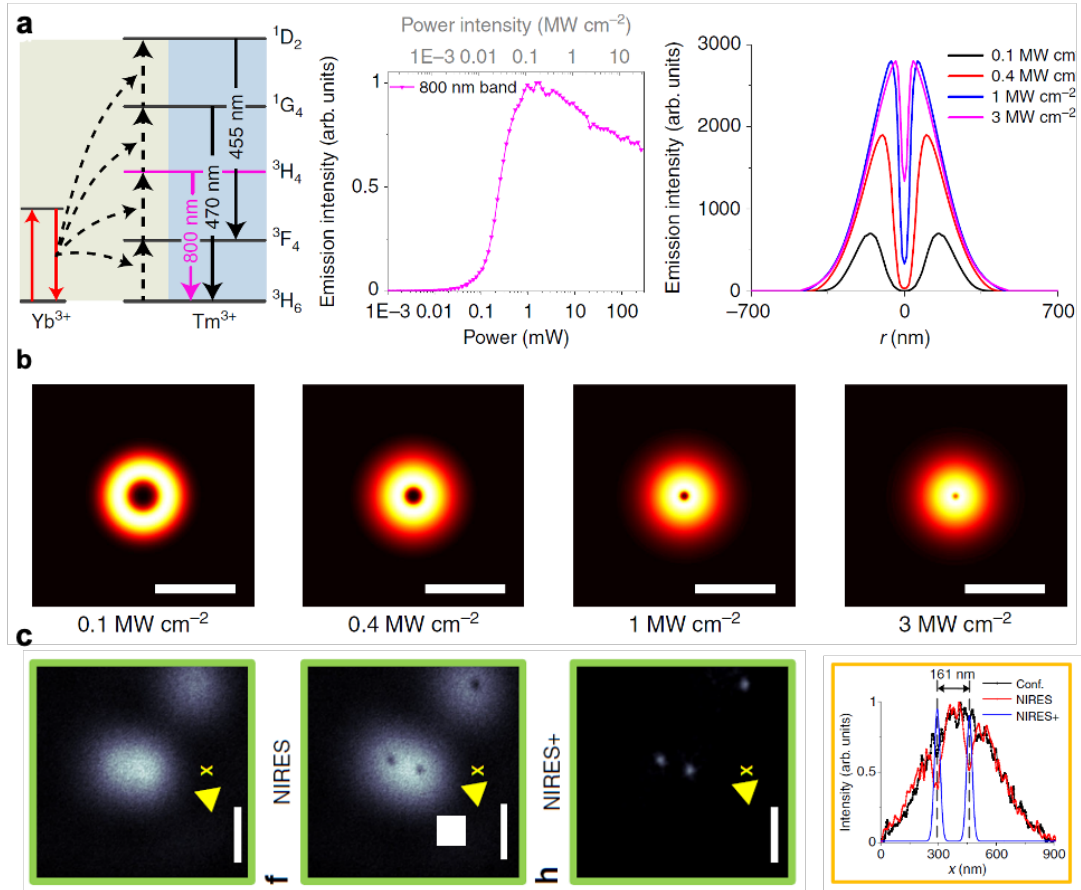


Fig. 1-17 NIRES nanoscopy based on UCNPs [97]. (a-b) The principle of NIRES nanoscopy using UCNPs as multiphoton probe for deep tissue imaging. **(c)** NIRES nanoscopy for super resolution imaging of single UCNPs through deep mouse liver tissue.

Additional innovations in this domain include excited-state absorption (ESA) and surface migration emission depletion (SMED) microscopy [98], as shown in Fig. 1-21. ESA-based depletion utilizes additional beams to selectively re-excite already excited electrons into higher non-emissive states, effectively reducing upconversion luminescence (UCL) from targeted regions. SMED, on the other hand, exploits energy

migration toward particle surfaces where nonradiative quenching occurs, especially useful in core-shell nanoparticle designs that spatially separate excitation and emission zones. Both techniques provide alternative mechanisms to suppress UCL with minimal hardware requirements and relatively low excitation intensities.

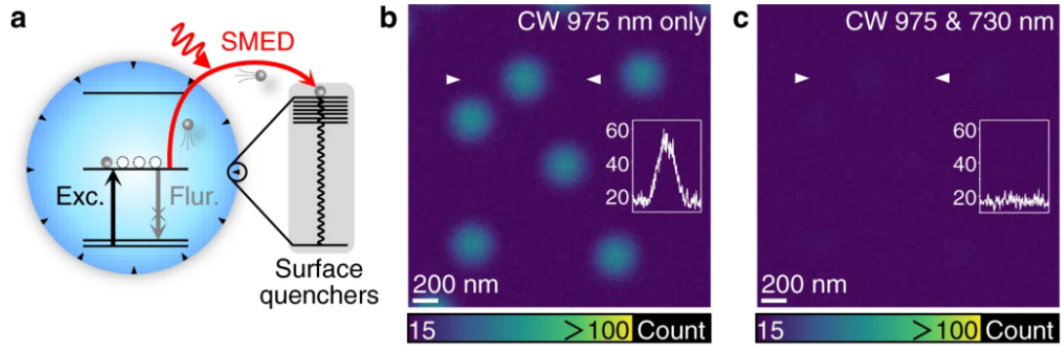


Fig. 1-18 SMED microscopy based on UCNPs [98]. (a) The proposed SMED mechanism. **(b-c)** Emission depletion of single NaGdF₄:Yb/Tm (18/0.3%) nanoparticles under dual-beam CW laser manipulation (975-nm excitation and 730-nm de-excitation).

UCNPs-based NSIM [99] uses patterned excitation light and benefits from the high-order nonlinearity of UCNPs to enhance spatial resolution (Fig. 1-22). For example, Yb³⁺/Tm³⁺-doped UCNPs exhibiting an emission slope of about four enable third-order or higher harmonic generation in the Fourier domain, increasing spatial resolution from roughly 300 nm to approximately 100 nm. By tuning the dopant ratio or shell design, the slope and emission brightness can be optimized for both lateral and axial resolution enhancement.

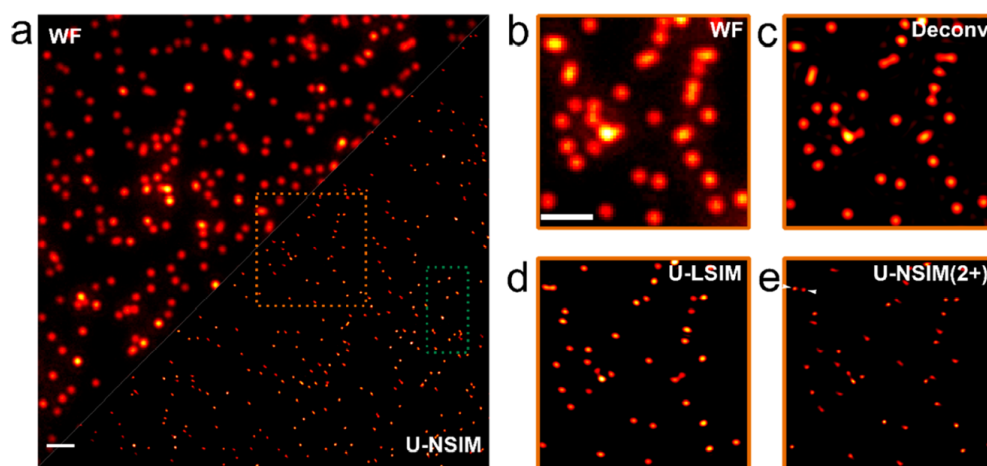


Fig. 1-19 Super-resolution imaging reconstructions by upconversion nonlinear SIM (U-NSIM) [99].

Super-linear microscopy is an emerging super-resolution imaging approach leveraging the intrinsic nonlinear emission behavior of UCNPs. Unlike traditional fluorescence microscopy, which is limited by diffraction to resolutions around half the excitation wavelength, super-linear microscopy exploits UCNPs' nonlinear response, wherein emission intensity increases disproportionately with excitation power, significantly narrowing the effective PSF. Denkova et al. demonstrated a three-dimensional sub-diffraction imaging method termed super-linear excitation-emission (SEE) microscopy by harnessing highly nonlinear emission from $\text{NaYF}_4: \text{Yb}^{3+}/\text{Tm}^{3+}$ nanoparticles [31], as shown in Fig. 1-23a. Remarkably, their technique enabled a resolution twice better than the diffraction limit in both lateral and axial directions under low-power CW near-infrared excitation, making it attractive for biological applications due to reduced phototoxicity and ease of implementation on conventional confocal microscopes. Building upon this, Wang et al. developed super-linear image scanning microscopy (SL-ISM) [100], further enhancing resolution by combining nonlinear UCNP emission with the image scanning microscopy approach, as shown in Fig. 1-23b. Using a low-power CW 976 nm excitation and detecting the 455 nm nonlinear emission from $\text{Yb}^{3+}/\text{Tm}^{3+}$ UCNPs, SL-ISM achieved a lateral resolution of approximately 120 nm, around 1/8 of the excitation wavelength. Moreover, they demonstrated a multifocal strategy to significantly speed up imaging while maintaining super-resolution capabilities,

emphasizing the practical potential of super-linear methods for routine bioimaging and deep-tissue applications. These advances highlight UCNP-based super-linear microscopy as a promising pathway toward accessible, high-resolution imaging technologies.

Photon avalanche (PA) is an emerging super-resolution imaging technique leveraging extreme optical nonlinearities inherent to certain UCNPs. In contrast to traditional multiphoton processes, photon avalanche exhibits a characteristic nonlinear emission response, where luminescence intensity rises steeply and disproportionately with excitation intensity once surpassing a certain threshold, driven by positive-feedback loops involving ESA and interionic cross-relaxation. Recently, Lee et al. demonstrated PA phenomena at room temperature in Tm^{3+} -doped UCNPs [101], achieving emission intensities scaling with approximately the 26th power of pump intensity, which enabled sub-70 nm spatial resolution under simple CW excitation (Fig. 1-25a). This capability is achieved due to exceptionally high excited-state absorption rates surpassing ground-state absorption by over four orders of magnitude, creating robust positive feedback and significantly lowering the power thresholds required compared to traditional nonlinear imaging modalities. Additionally, Dong et al. reported parallel photon avalanche (PPA) in Ho^{3+} -doped UCNPs [102], where dual reservoir energy levels ($^5\text{I}_7$ and $^5\text{I}_6$) simultaneously activate multiple emission bands (red, green, and blue). This parallel mechanism allows tunable emission chromaticity and multicolor sub-diffraction imaging with lateral resolutions of approximately 78–102 nm under modest CW excitation powers ($\sim 22 \text{ kW/cm}^2$). Such developments highlight UCNP-based photon avalanche nanoscopy as an attractive super-resolution modality due to its extraordinary resolution, low phototoxicity, and compatibility with existing microscopy platforms.

Technique	Principle	Resolution	Excitation	Advantages	Limitations
STED	Combines excitation and depletion beam	~28-66 nm	Dual CW excitation	High resolution, widely used	Dual-beam alignment, phototoxicity, complex
FED	Subtracts images acquired with solid vs. donut beams;	~170 nm	Sequential or dual CW lasers	No bleaching, simple, no depletion	Resolution limited, sensitive to SNR
NIRES	Based on saturation of multiphoton UCNPs emission	~34 nm	Doughnut CW NIR excitation	Deep penetration, low phototoxicity	Higher power, moderate resolution, alignment of doughnut beam
SMED	Depletion of surface migration states suppresses emission	~16.8 nm	Dual CW beams	Low inhibition saturation intensity via surface migration	Complex dual-beam setup, material dependent, dual-beam setup
NSIM	Combines structured illumination with nonlinear UCNPs emission	~100-130 nm	CW structured illumination	Straightforward setup, simple	Limited resolution, limited power density, limited FOV
SEE	Exploits highly super-linear UCNPs emission confined to the focal center	~216 nm	Single CW excitation	No depletion beam, low phototoxicity, compatible with standard confocal	Requires specially engineered UCNPs, limited resolution
PA	Photon avalanche effect	~60-80 nm	Single CW excitation	Extreme nonlinearity; very high resolution with simple beam path	Special dopants, threshold control, complex, unstable

Table 1-2 Comparison of UCNPs-based super-resolution microscopy techniques.

1.4 Thesis Outline

The primary objective of this thesis is to systematically advance the frontiers of UCNPs-based deep-tissue and super-resolution optical imaging by leveraging the unique nonlinear optical responses, dual-emission capabilities, and exceptional

photostability under CW excitation. Through synergistic integration of innovative nanomaterial design, advanced optical engineering, and customized computational algorithms, this work addresses persistent limitations of conventional nonlinear microscopy in complex biological environments, such as high excitation thresholds, insufficient penetration depth, and diffraction-limited spatial resolution. The thesis is organized as follows.

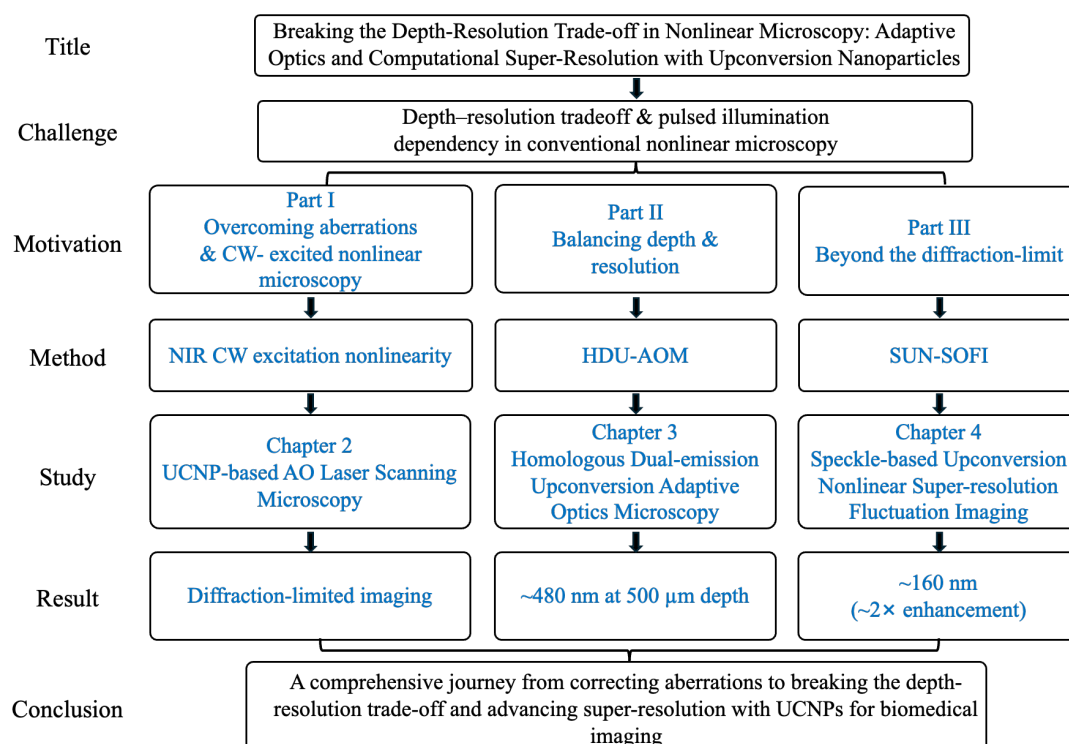


Fig. 1-20 Thesis outline. Illustrating the research title, challenge, motivation, method, study, result, and conclusion.

Chapter 1 begins with an introduction to fundamental optical microscopy techniques, outlining their principles and development for biological imaging. This is followed by an overview of AO technology, emphasizing its role in correcting optical aberrations and improving imaging performance and extending imaging depth in scattering biological tissues. Next, the chapter presents the unique photophysical properties and nonlinear optical mechanisms of UCNP, positioning them as advanced nonlinear probes for modern microscopy. The subsequent sections review recent advances in the application of UCNP for bioimaging, including their advantages for deep-tissue

imaging and multiplexed detection. Finally, the chapter summarizes the state-of-the-art in UCNP-based super-resolution imaging, highlighting key breakthroughs. By providing a coherent introduction to these technologies and their integration, the chapter establishes the scientific motivation and framework for the subsequent research presented in this thesis.

Chapter 2 details the design, construction, and optimization of advanced AO microscopy systems that leverage UCNPs as both imaging and direct wavefront GS. This chapter systematically describes the key principles, experimental setup, and technical innovations involved in building the UCNP-based AO imaging platform, addressing critical factors such as system integration, optical alignment, aberration correction, and performance evaluation. The chapter lays the technical foundation for Chapter 3 for robust, dual-emission deep-tissue imaging applications.

Chapter 3 builds upon the AO microscopy system established in Chapter 2 and investigates strategies that utilize the dual-emission properties of UCNPs to further enhance nonlinear imaging depth and spatial resolution. This chapter systematically examines how dual-emission UCNPs can be integrated with AO techniques under CW excitation to facilitate robust high-resolution deep-tissue imaging. Through a combination of system optimization, experimental validation, and comparative analysis, this chapter demonstrates expanded potentials of UCNP-based imaging platforms for advanced biomedical applications.

Chapter 4 presents super-resolution imaging using SOFI techniques adapted for non-blinking UCNPs. This chapter explores how speckle-based SOFI methodologies can be innovatively applied to UCNPs, which inherently lack fluorescence blinking, by introducing controlled excitation modulation and statistical analysis of emission fluctuations. Through theoretical modeling, system implementation, and experimental demonstrations, this chapter establishes a robust framework for achieving stable super-resolution imaging in challenging biological environments, thereby expanding the versatility and applicability of UCNP-based super-resolution techniques.

Chapter 5 provides a comprehensive summary of the main research achievements presented throughout the thesis, synthesizing key results from the development of deep-tissue and super-resolution imaging techniques based on UCNPs. This chapter discusses the primary challenges and limitations encountered during the research and offers a critical evaluation of the current state and future prospects of UCNP-enabled bioimaging. Finally, this chapter outlines potential directions for further studies, highlighting opportunities for continued innovation and translation of UCNP-based imaging technologies in biomedical research and clinical applications.

2 UCNP-BASED DIRECT WAVEFRONT SENSING NONLINEAR LASER SCANNING MICROSCOPY

2.1 Overview

There are two primary sources of aberration in nonlinear laser scanning microscopy (NLSM): 1) system aberrations arising from minor imperfections in optical components, misalignments within the microscope, and refractive index mismatches of the cranial window's cover glass; 2) sample aberrations caused by refractive index inhomogeneities in brain tissue or sample preparation and tissue scattering. These aberrations distort the wavefront of the excitation beam, enlarging the focal spot and reducing focal intensity (Fig. 1-5), thereby degrading spatial resolution and signal-to-background ratio.

The direct AO method offers a robust and efficient approach for correcting these aberrations. In direct AO, a DM or SLM is inserted into the imaging path to correct phase distortions based on real-time wavefront measurements (Figs. 1-8 & 1-10). A SHWS measures the wavefront emitted by an embedded GS, which serves as an ideal point source within the sample. As the emitted light traverses through both biological tissues and optical components, it accumulates aberrations from the sample and imaging system. The SHWS quantitatively captures these combined aberrations, generating a precise phase map used to control the DM or SLM, thus restoring the excitation beam to its optimal wavefront and ensuring diffraction-limited imaging performance.

In this chapter, we apply direct AO to UCNPs-based NLSM. First, we excite UCNPs and record the emission signal by a detector; next, we use the SHWS to obtain the GS and then calculate the wavefront distortions; finally, we load the computed phase map onto the DM and get a corrected and enhanced signal by the detector. This synchronous correction of both system and sample aberrations significantly enhances spatial resolution and SNR for large-aberrations samples, offering an effective strategy for

deep-tissue imaging.

2.2 Optical Setup

Figure 2-1 shows the optical layout of the UCNP-based AO-NLSM system. A CW 980 nm laser beam is expanded and collimated by lenses L_1 and L_2 into an 8 mm parallel beam. The beam is reflected by a DM for pre-correction, passes through relay lenses L_3 and L_4 , and is directed by a dichroic beamsplitter (BS_2) onto the X-galvo scanner. It then travels through another relay lens pair (L_5 and L_6) onto the Y-galvo scanner, after which it is relayed by lenses L_7 and L_8 and redirected by a steering mirror (M) into the objective lens (OL). The OL focuses the beam onto the sample, performing point-by-point raster scanning.

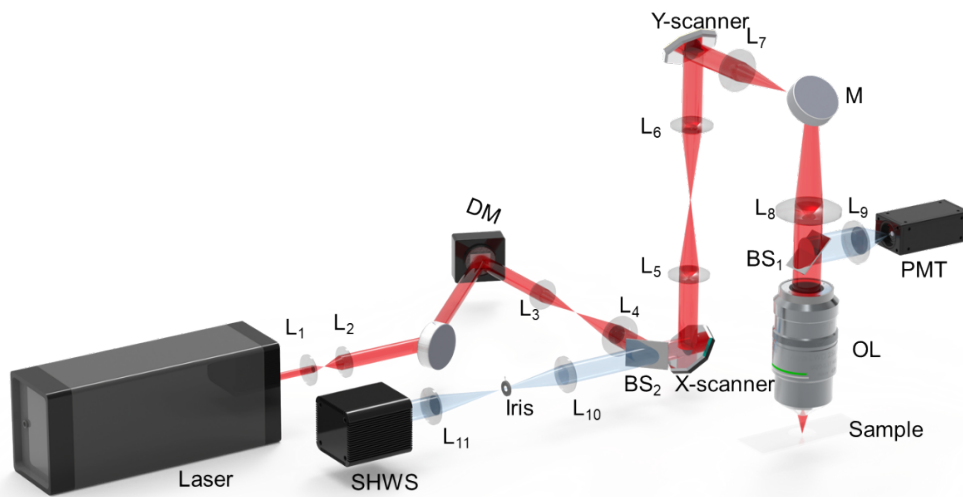


Fig. 2-1 The optical setup of direct wavefront sensing AO-NLSM. L: lens; BS: beam splitter; DM: deformable mirror; OL: objective lens; PMT: photomultiplier tube.

Subsequently, UC fluorescence excited at the OL focus is recollected by the OL and separated into two paths by beamsplitter BS_1 . One reflected portion is focused by lens L_9 onto a PMT for imaging detection, while the other transmitted portion retraces the excitation path in reverse. This transmitted portion passes back through two relay lens pairs (L_7 – L_8 and L_5 – L_6), undergoing descanned via the X&Y-galvo scanners. After passing through beamsplitter BS_2 , it is relayed by lenses L_{10} and L_{11} into the SHWS,

which measures emission wavefront distortions. Real-time control algorithms reconstruct the aberration profile from SHWS measurements and apply corresponding corrections to the DM, restoring diffraction-limited imaging performance.

To facilitate real-time correction of optical aberrations, we constructed a home-built direct-wavefront-sensing AO kit, as illustrated in Fig. 2-2. This AO system primarily comprises a DM (DM97-15, ALPAO), a SHWS assembled from a sCMOS camera (Dhyana 400BSI, Tucsen) and a precisely fabricated microlens array (64-483, Edmund). In operation, the SHWS accurately measures incoming wavefront distortions, enabling subsequent feedback-driven deformation of the DM surface to precisely compensate for the measured aberrations. By operating in a closed-loop configuration, this AO setup continuously refines wavefront quality, thereby restoring diffraction-limited imaging conditions under various experimental scenarios.

Home-built AO kit

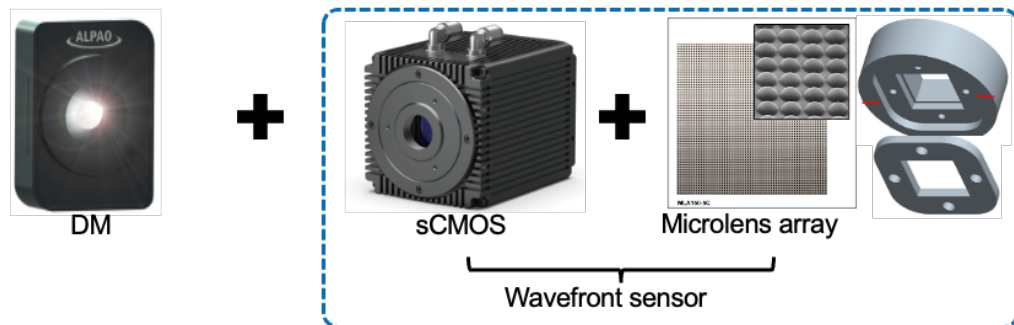


Fig. 2-2 Schematic illustration of our home-built direct-wavefront-sensing adaptive optics (AO) kit. The AO system consists of a DM and a SHWS, which is assembled by integrating a sCMOS camera and a microlens array.

2.3 DM Calibration

DMs inherently exhibit static surface errors arising from fabrication and assembly processes [103]. Manufacturing tolerances in the reflective faceplate, such as thickness non-uniformity and residual polishing errors, introduce nanometer-scale height deviations across the mirror surface. Each actuator's displacement also elastically couples to neighboring regions, producing complex influence functions rather than perfectly localized deflections. Moreover, bonding the faceplate to its substrate and

pre-loading during assembly generate residual stresses that manifest as additional static astigmatism, coma, or higher-order deformations. Even after careful alignment, these zero-voltage “flat” errors can exceed tens of nanometers RMS, as demonstrated in X-ray DM flattening experiments reaching 0.7 nm RMS only after active compensation [104]. Characterization efforts in astronomical AO systems further report that uncorrected static errors limit open-loop performance to well above desired sub-nanometer levels without pre-flattening.

If left uncorrected, these intrinsic deformations consume a significant portion of the DM’s dynamic stroke and reduce its linear response to external aberrations. In practice, the DM would need to allocate driver voltage to cancel both its own static figure error and the sample- or system-induced aberrations simultaneously, thereby compromising correction range and accuracy. To prevent this, a “flat map” is generated prior to routine use: one measures the DM’s zero-drive wavefront with an interferometer or SHWS, then computes and applies a reverse-bias voltage pattern that cancels the static error. This pre-compensation ensures that subsequent adaptive corrections target only dynamic aberrations, maximizing available stroke and preserving linearity and fidelity in high-order wavefront control [105,106].

2.3.1 Optical Setup

Figure 2-3 depicts the layout used to characterize and pre-flatten the DM. A CW laser source is first expanded by a beam expander to produce a collimated beam whose diameter slightly overfills the DM aperture. This ensures that all actuators on the mirror surface receive uniform illumination. After reflection from the DM, the beam returns along its incoming path to a non-polarizing beamsplitter. A fraction of this reflected light is then directed through a beam expander, which adjusts the beam size to optimally match the entrance pupil of the SHWS.

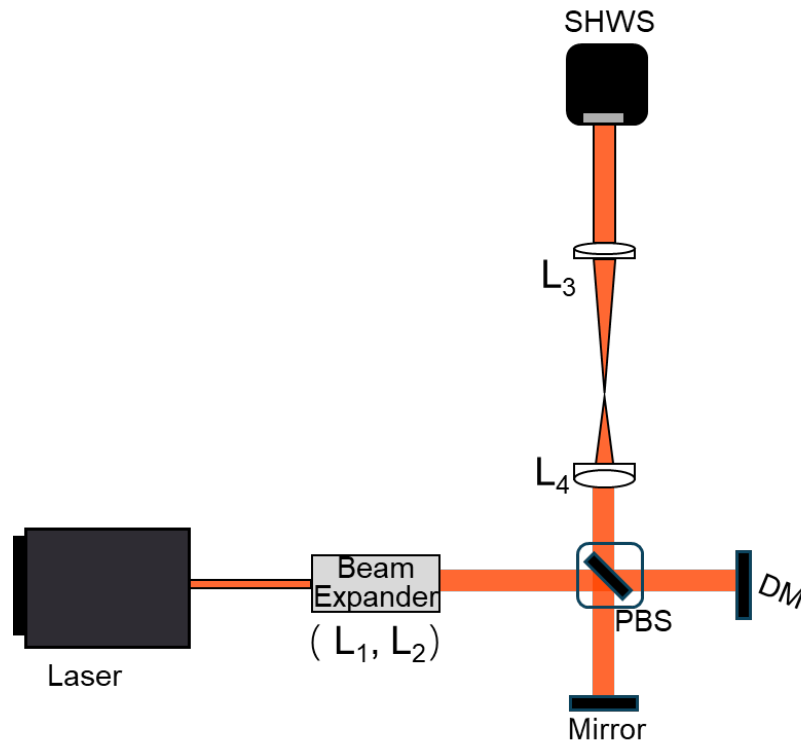


Fig. 2-3 Optical setup for DM flat-map characterization. A CW laser is expanded by Beam Expander 1 to overfill the DM. Reflected light from the DM returns through a non-polarizing beamsplitter and is resized by Beam Expander 2 before reaching the SHWS. A reference flat mirror can be inserted at the pupil plane to switch between system-level and DM-only wavefront measurements.

The SHWS captures the residual wavefront error introduced by the DM's intrinsic surface figure, resolving local slopes across each sub-aperture. A reference flat mirror in the transmission arm maintains alignment and allows switching between system-level and DM-only measurements. By comparing the measured wavefront to an ideal plane, which is measured by the reflective mirror, one obtains the static surface deformation map of the DM. This optical arrangement provides the high spatial sampling and phase accuracy required to compute the “flat-map” compensation voltages that pre-correct the DM before dynamic AO operation.

2.3.2 Calibration Results

We found that our DM exhibited seriously static surface errors arising from fabrication

and assembly processes. The experiment result we performed to evaluate the intrinsic surface quality of DM prior to any calibration is as shown in Fig. 2-4. A single-frequency 632.8 nm He–Ne laser was expanded to a 6 mm collimated beam and sequentially reflected by two optics placed in the same kinematic mount: a $\lambda/10$ flat reflective mirror and the DM under test. The reflected beams propagated 3 m to an observation plate and then were captured by the phone’s camera (Fig. 2-3). The left-hand spot in Fig. 2-4 corresponds to the reference reflective mirror and appears as an almost perfect Airy disc, confirming that the laser, alignment, and detection chain are essentially diffraction-limited. When the reference flat was replaced by the DM, however, the recorded far-field profile (right in Fig. 2-4) became highly distorted: instead of a compact central lobe, the intensity redistributed into a broken annulus with multiple bright spikes. This morphology indicates the presence of large low-order aberrations (dominated by astigmatism and coma) superimposed on mid-spatial-frequency errors.

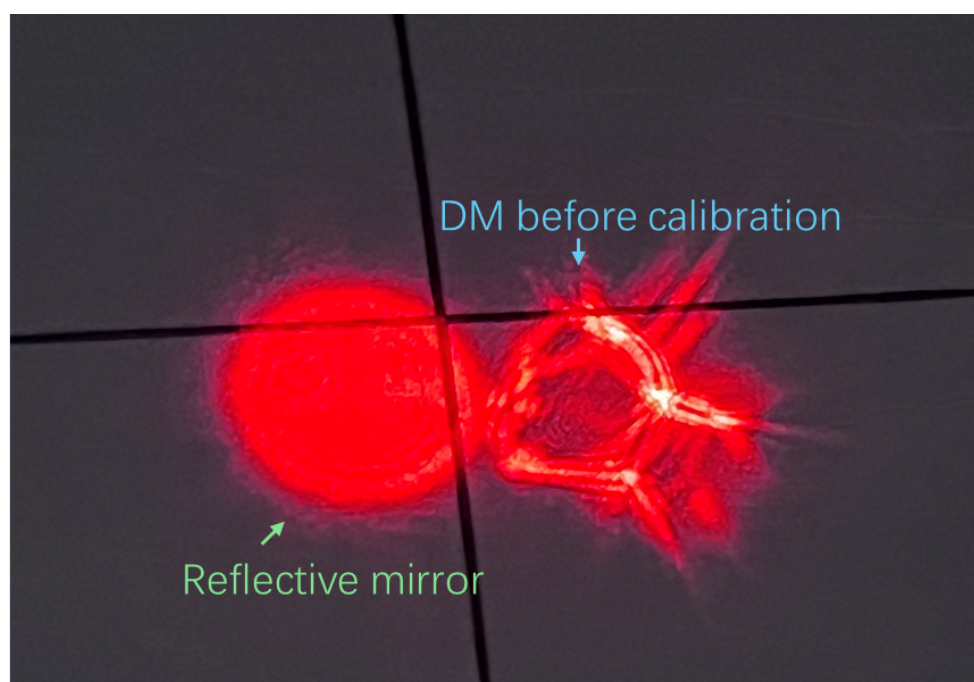


Fig. 2-4 Far-field beam profiles recorded during flat-map characterization of the DM. A collimated laser beam was sequentially reflected by a $\lambda/10$ reference flat reflective mirror (left, green) and the uncalibrated DM (right, blue).

We then calculated the static phase error of the DM based on the optical setup in Fig. 2-3. The measured static phase results in DM's zero-bias state is presented in Fig. 2-5, which is plotted as a function of actuator index. Each bar corresponds to the phase shift induced by one actuator when all others are held at zero voltage. The distribution spans roughly ± 0.25 a.u., with the largest negative deviation near actuator 95 (-0.23 a.u.) and the largest positive deviation near actuator 94 ($+0.2$ a.u.). Most actuators introduce errors within ± 0.1 a.u., but a small number lie outside this range, highlighting nonuniformities in the faceplate response.

The circular inset shows the two-dimensional reconstruction of the DM surface error obtained by superposition of all individual actuator influence functions. Regions of both positive and negative curvature are evident, indicating residual astigmatism and higher-order surface figure distortions. These per-actuator phase measurements form the rows of the influence-matrix $\mathbf{H}$, which is inverted to compute the flat-map compensation voltages. Applying this flat map prior to dynamic operation cancels the static errors shown here, ensuring that the mirror's full stroke is available for real-time correction of sample- and system-induced aberrations.

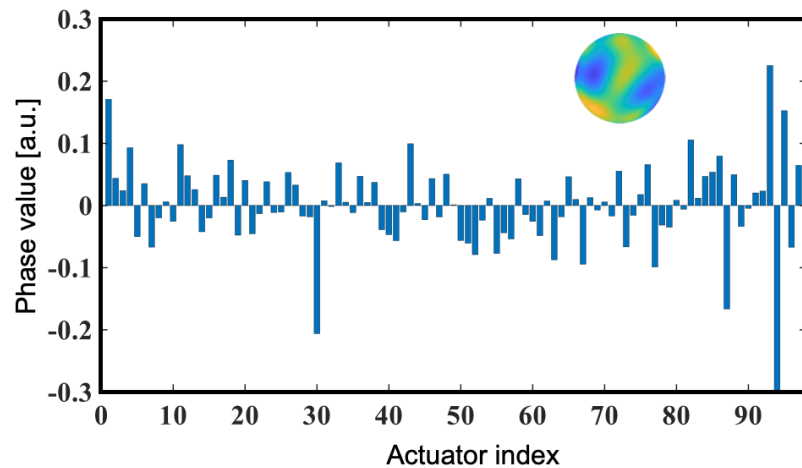


Fig. 2-5 Static surface error of the deformable mirror. Bar plot of the uncorrected zero-bias phase deformation measured for each DM actuator, with actuator index on the horizontal axis and phase error (arbitrary units) on the vertical axis. The circular inset shows the two-dimensional reconstruction of the DM surface error obtained by superposing the individual actuator influence functions.

After uploading the pre-compensation phase map that we computed for the DM, the far-field beam profile changes markedly as displayed in Fig. 2-6. The optical path length, collimated beam diameter and camera settings are identical to those used for Fig. 2-4, so that the observed differences originate solely from the flat-map calibration. The left spot, produced by a $\lambda/10$ reference flat reflective mirror, remains an almost perfect Airy disc and serves as the diffraction-limited benchmark. The right spot, reflected from the calibrated DM, has collapsed into a compact and nearly axis-symmetric disc in sharp contrast to the fragmented annulus recorded before calibration; only minor granular noise is visible, and this is attributed to residual surface distortion. These results confirm that the calibration process using the optical setup in Fig. 2-3 effectively removes the DM surface-induced error and supplies an optical quality surface suitable for direct AO correction of specimen-induced aberrations in subsequent microscopy experiments.

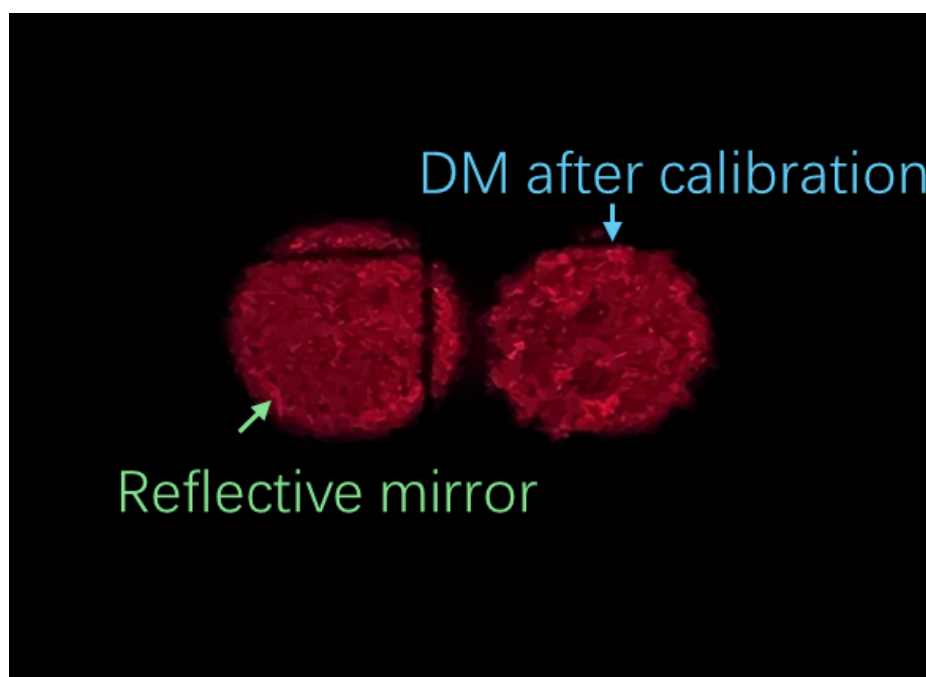


Fig. 2-6 Far-field beam profiles acquired after loading the computed pre-compensation phase map onto the DM. The $\lambda/10$ reference flat mirror (left, green) yields a diffraction-limited Airy pattern that serves as the baseline. The calibrated DM (right, blue) now returns an almost circular and symmetric intensity distribution, demonstrating that the flat-map voltages effectively suppress the low- and mid-spatial-frequency aberrations observed before calibration.

2.4 AO Algorithm and Control Program

2.4.1 Principle of UCNPs-based AO

Calibration between DM and SHWS. The system aberration resulting from the misalignment of lenses was compensated before the DM-SHWS calibration. This aberration was pre-compensated using the methods outlined in Reference [107]. The measured results showed that the peak-to-valley (PV) value of the aberration was lower than 0.1 μm , which did not cause any signal and resolution degradation. Consequently, this aberration was disregarded in subsequent steps.

The calibration process between the DM and the SHWS aimed to obtain a transmission matrix, denoted as M_{SH2DM} . This matrix establishes a relationship between spots shift in SHWS and the actuator movement in DM. Initially, a flat mirror was positioned at the back pupil plane of the objective, thereby linking the DM and the SHWS in the laser path. As a result, the DM, the pupil plane, and the lens array plane of SHWS are mutually conjugated. Subsequently, the DM flat command was applied to generate a plane wavefront, which in turn generated a reference spot array in SHWS. The spot locations of the array are represented as $S_{ref} = (x_1 \dots x_N, y_1 \dots y_N)^T$, where x_i and y_i denote the centroid location of the spot i . Third, 5000 DM test patterns, encompassing single-axis changes and multiple-axis changes with Zernike patterns, were recorded along with their corresponding spot locations in the SHWS. The movements of DM actuators for each pattern were denoted as $\Phi = (a_1, a_2, \dots, a_{140})^T$, where a_k is the normalized movement of actuator k . The spot locations of SHWS for these patterns were denoted as $S = (x_1 \dots x_N, y_1 \dots y_N)^T$. Lastly, M_{SH2DM} was solved from Eq (2-1) using the least-squares method:

$$\Phi = M_{SH2DM} (S - S_{ref}), \quad (2-1)$$

where Φ represents the movements of actuators of test patterns, and S represents the spot locations of SHWS corresponding to the test patterns. Note that M_{SH2DM} was used to calculate the aberration of the fluorescence. Hence, the system must be carefully

calibrated to ensure the laser that reflected from the back pupil of the objective have the same emit angle and the same size as the fluorescence that collected from the focal point of the objective[108].

Sample aberration measurement. Prior to conducting the sample aberration measurement, it is imperative to acquire a fluorescent SHWS reference, denoted as $\mathbf{S}_{F-ref}$, from a sample that is devoid of aberrations. Under ideal circumstances, the laser reference spot diagram ($\mathbf{S}_{ref}$) and the fluorescence reference spot diagram ($\mathbf{S}_{F-ref}$) should be congruent. This congruence is crucial as we employ the transmission matrix ($\mathbf{M}_{SH2DM}$), which is measured by the laser, to compute the wavefront aberration of fluorescence. Nevertheless, due to factors such as system chromatic aberration, there exists a pixel offset ranging from 0 to 5 between the two diagrams. Consequently, it becomes necessary to measure the fluorescence reference point diagram ($\mathbf{S}_{F-ref}$) independently.

During the process of measuring the sample aberration, the laser was scanned within a confined FOV ($25 \mu\text{m} \times 25 \mu\text{m}$). An SHWS background was obtained by turning the objective lens switch off (no light passing through the objective), and subsequently, a fluorescent SHWS image was captured by turning the objective lens switch on. The subtraction of the background from the SHWS image aids in the suppression of ambient noise. Following this, the spot locations of the background-subtracted SHWS image, denoted as $\mathbf{S}_F$, were calculated by the centroid algorithm. Finally, the actuator movements of the DM, represented as Φ_F , could be computed as Eq. (2-2):

$$\Phi_F = \mathbf{M}_{SH2DM}(\mathbf{S}_F - \mathbf{S}_{Fs-ref}) \quad (2-2)$$

2.4.2 Control Program

Microscope timing, scanning and actuator control are performed using the open-source ScanImage platform (Vidrio Technologies) [109]. ScanImage operates as the central application responsible for coordinating laser modulation, galvanometer steering, data acquisition and hardware synchronization. AO functionality is integrated by developing custom user functions in MATLAB that augment ScanImage's native callback

framework.

Aberration measurement is carried out within an acquisition callback that invokes the SHWS readout immediately after each image frame. The raw sensor centroids are fed into a MATLAB routine that reconstructs the current wavefront error via a modal decomposition. A subsequent callback translates the reconstructed phase map into actuator drive voltages by applying the inverse of the precomputed flat-map influence matrix. These voltages are transmitted to the deformable mirror controller over MATLAB's serial interface prior to the next scan.

By leveraging ScanImage's real-time scripting interface, wavefront measurement, phase reconstruction, voltage computation and mirror loading all occur within the standard frame interval. This seamless integration permits closed-loop correction of dynamic aberrations on a frame-by-frame basis without incurring additional hardware latency, thereby enabling high-speed laser scanning fluorescence imaging in scattering media.

2.5 System Alignment

Accurate aberration correction requires that the X-scanner, Y-scanner, DM and SHWS all be precisely conjugated to the rear pupil plane of the objective. Misalignment among these elements degrades wavefront-sensor accuracy and reduces the deformable mirror's effective stroke. Our alignment protocol consists of two stages: bulk optical conjugation and fine spot-array alignment with aperture matching.

2.5.1 Bulk Optical Conjugation

First, each element is placed in a dedicated 4-f relay so that its active aperture is conjugated onto the rear pupil of the objective lens. The DM is mounted on a X-&Y-translation stage. As shown in the optical setup (Fig. 2-1), lenses L_3 and L_4 form the first 4-f relay that conjugates the DM to the pupil. The X-scanner and Y-scanner are subsequently inserted into their respective 4-f relays formed by L_5/L_6 and L_7/L_8 . Finally, a third relay of L_9 and L_{10} conjugates the SHWS microlens array to the same pupil plane.

In this configuration, any phase correction applied by the DM maps directly to the SHWS measurement.

2.5.2 Spot-Array Alignment and Aperture Matching

To refine alignment, we compare spot grids in two modes. A flat mirror under the objective produces a back-reflected laser grid (Fig. 2-7b). We select well-spaced laser spots and record their centroids to establish a reference lattice. Next, we replace the mirror with the fluorescent sample and adjust the objective focus until the fluorescence spot grid (Fig. 2-7a) coincides with the reference lattice. Switching back to the mirror and remeasuring the laser grid allows a second fluorescence focus adjustment. Repeating this cycle converges on perfect coincidence of fluorescence and laser spot arrays, as shown in Fig. 2-7a.

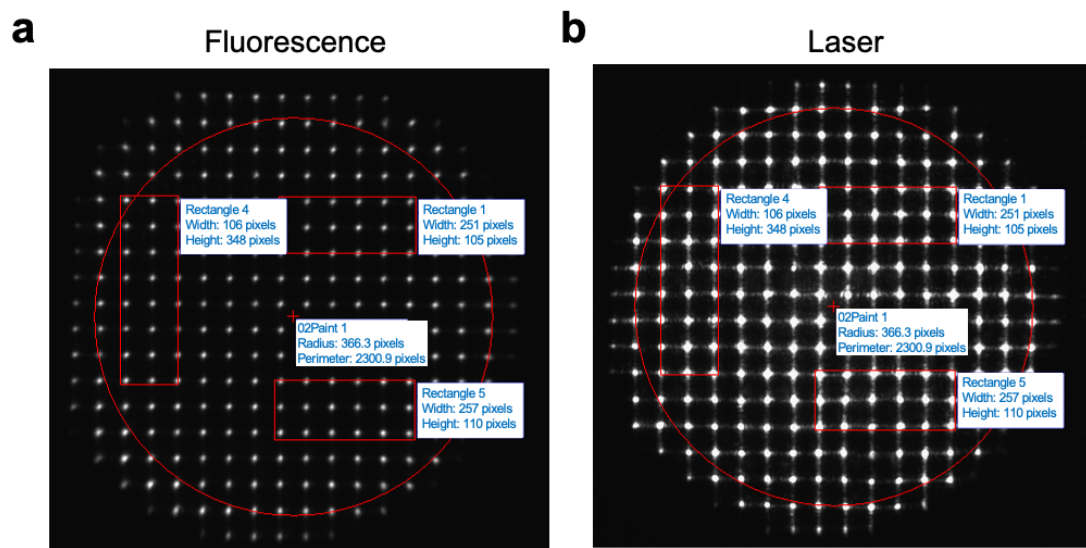


Fig. 2-7 Spot-array measurement in fluorescence and laser modes. (a) Fluorescence-excited spot grid recorded by the SHWS. The red solid circle denotes the nominal pupil boundary, and the red measurement rectangles and blue texts mark regions of interest with pixel width, height and area annotations. **(b)** Back-reflected laser spot grid under identical optical conjugation. The same measurement regions (red marked) are overlaid with their dimensions displayed.

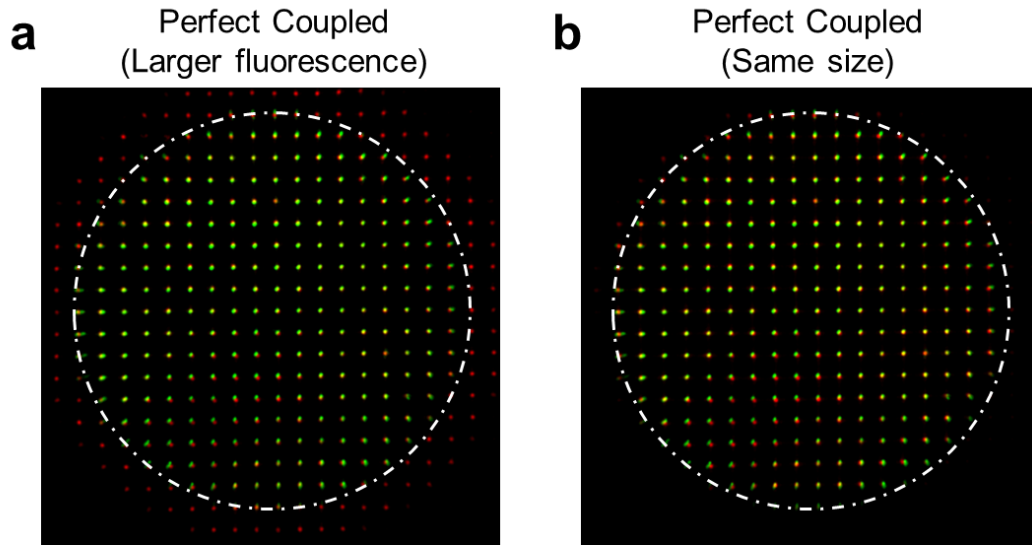


Fig. 2-8 Perfect coupling of fluorescence and laser spot arrays. (a) Overlay of fluorescence emission (green) and back-reflected laser (red) spots before aperture matching, showing larger fluorescence spots within the nominal pupil (dashed white circle). (b) Overlay after inserting and adjusting an iris in the SHWS path to equalize spot diameters, resulting in co-registered fluorescence (green) and laser (red) spots of identical size.

After positional alignment, fluorescence spots (green) appear larger than laser spots (red) (Fig. 2-8a). We therefore introduce an adjustable iris before the SHWS and narrow it until the fluorescence grid is clipped to the same diameter as the laser grid. This produces spot arrays that coincide in position and match in size, as demonstrated in Fig. 2-8b. Ensuring identical spot diameters is essential because all subsequent DM-SHWS calibration and wavefront correction use the laser reflection path; the fluorescence path does not traverse the deformable mirror and must therefore be aperture-matched for consistent calibration.

2.6 AO Calibration Results

2.6.1 Spot Diagrams Recognition

Accurate mapping between DM actuators and SHWS spots array is fundamental to influence-matrix calibration. We adapted the streamlined workflow described in Yao et al.[108] for robust spot detection, indexing and uniformity assessment.

First, a reference SHWS image is acquired by SHWS under zero DM bias (after correction) when placing a reflective mirror on the pupil-conjugated plane. Raw frames undergo background subtraction and band-pass filtering to isolate high-contrast spot features. Candidate centroids are then detected via a circular Hough transform tuned to the lenslet focal spot size (Fig. 2-9a). We constrain detections within the pupil boundary to exclude stray reflections or edge artifacts.

Next, each centroid is enclosed in a fixed-size square box and assigned a unique actuator index by fitting the detected points to a regular 2D lattice (Fig. 2-9b). We follow the lattice-fitting and outlier-rejection approach to ensure one-to-one correspondence between spots and DM actuators. An inset in Fig. 2-9b confirms that each box fully contains its corresponding spot.

To verify that every spot has been correctly localized, we overlay the computed centroids (red dots) onto their corresponding bounding boxes (yellow) within the nominal pupil (yellow circle) in Fig. 2-9c. In this view each red centroid lies at the geometric center of its yellow square, confirming that the center-of-gravity algorithm has correctly identified the true spot centers and that no actuators have been missed or misindexed across the full aperture.

Finally, we assess spot size by computing the radial intensity profile in each yellow box and extracting the half-maximum radius. When plotted against actuator index (Fig. 2-9d), these radii cluster tightly around 47 pixels with a variation of approximately ± 3 pixels. This degree of uniformity confirms that all sub-apertures produce spots of nearly identical diameter, a prerequisite for reliable wavefront reconstruction.

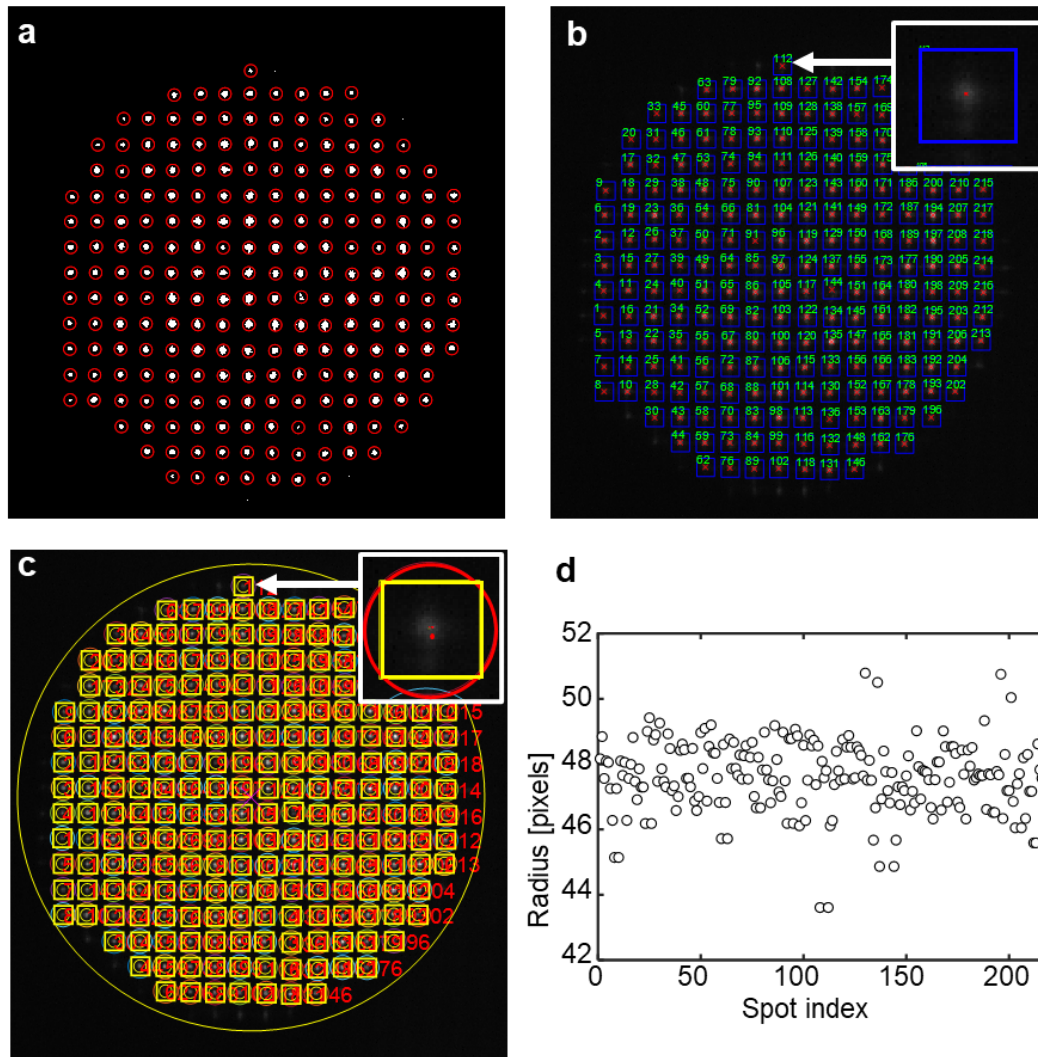


Fig. 2-9 AO calibration: SHWS spot detection and uniformity analysis. (a) Raw SHWS image under uniform fluorescence illumination, with red circles indicating spot centroids detected by the center-of-gravity algorithm. (b) Indexed spot grid: blue squares mark fixed-size bounding boxes around each centroid and green numbers of label actuator indices determined by fitting to a 2D lattice. Inset (white frame) shows a single spot at higher magnification. (c) Overlay of centroids (red circles) and indexed boxes (yellow squares) within the pupil boundary (yellow circle). Inset (white frame) provides a zoomed view of one boxed spot. (d) Half-maximum radii measured from each boxed region, plotted as a function of DM actuator index.

2.6.2 AO Calibration

AO calibration is carried out by sequentially stimulating each deformable mirror actuator and measuring its effect on the SHWS spot pattern. A single actuator is selected and driven with a small voltage increment while all other actuators remain at their bias setting shown in Fig. 2-10a. The Shack Hartmann sensor acquires two wavefront slope images, one immediately before and one immediately after the voltage step. Spot centroids in each image are located by a center of gravity algorithm and their horizontal and vertical displacements are computed. These displacement vectors capture the local change in wavefront slope produced by the stimulated actuator.

The displacement vectors for one actuator form a complete map of spot shifts across the pupil and are plotted as a vector field in Fig. 2-10b. This measurement is then repeated for every actuator in numerical sequence. The collection of displacement maps is assembled column by column into the influence matrix $\mathbf{H}$, where each column describes how a unit voltage applied to a single actuator affects the slope at every SHWS sub-aperture.

Once the influence matrix $\mathbf{H}$ is populated, it is processed by a regularized inversion routine to compute its pseudoinverse. This operation mitigates noise amplification and ensures numerical stability when solving for corrective commands. During live imaging, measured wavefront errors are projected through the pseudoinverse of $\mathbf{H}$ to yield the optimal set of actuator voltages that correct sample induced aberrations.

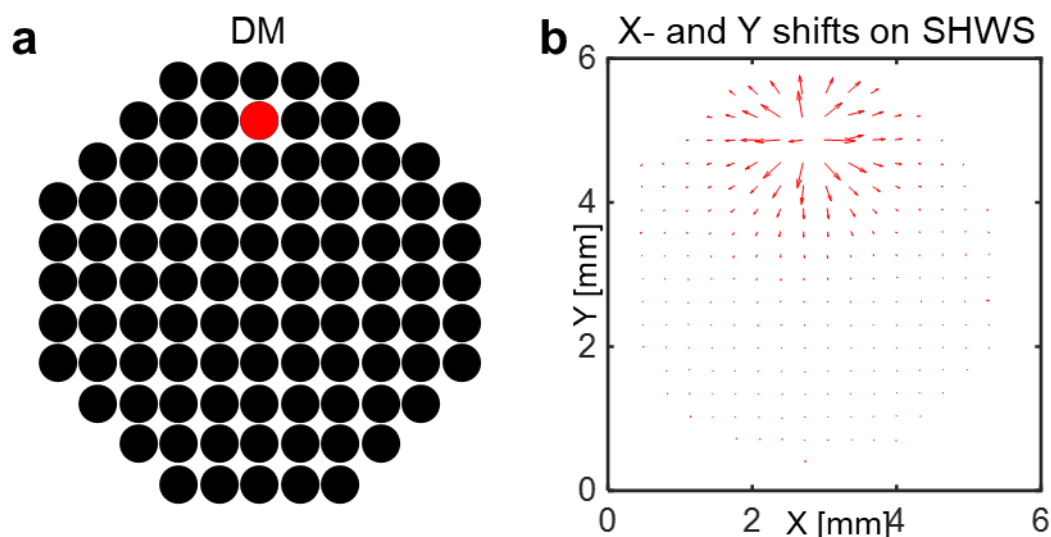


Fig. 2-10 AO calibration by actuator poke and spot shift measurement. (a) Schematic of the deformable mirror actuator array with a single actuator highlighted in red for calibration. (b) Measured X and Y displacements of SHWS focal spots induced by the highlighted actuator, plotted as a vector field over the pupil plane.

2.6.3 Measurement Verification

To verify the accuracy of the measured DM-SHWS transmission matrix we employed two complementary tests: a modal test using Zernike polynomials and a single-actuator poke test.

The modal test was conducted by sequentially displaying individual Zernike modes on the deformable mirror and measuring the resulting wavefront with the SHWS. For the seventh-order Zernike mode, the measured wavefront was compared against the commanded value, with the loaded RMS amplitude indicated by orange bars and the corresponding amplitude reconstructed from SHWS measurements represented by green bars, as shown in Fig. 2-11a. This approach was further extended to simultaneously loaded combinations of Zernike modes, specifically including third-, sixth- and ninth-order modes. Again, the loaded and reconstructed amplitudes are represented by orange and green bars, respectively, as illustrated in Fig. 2-11b. The modal test results demonstrated high fidelity, with reconstructed modal amplitudes closely matching the commanded values for both single-mode and multi-mode

scenarios.

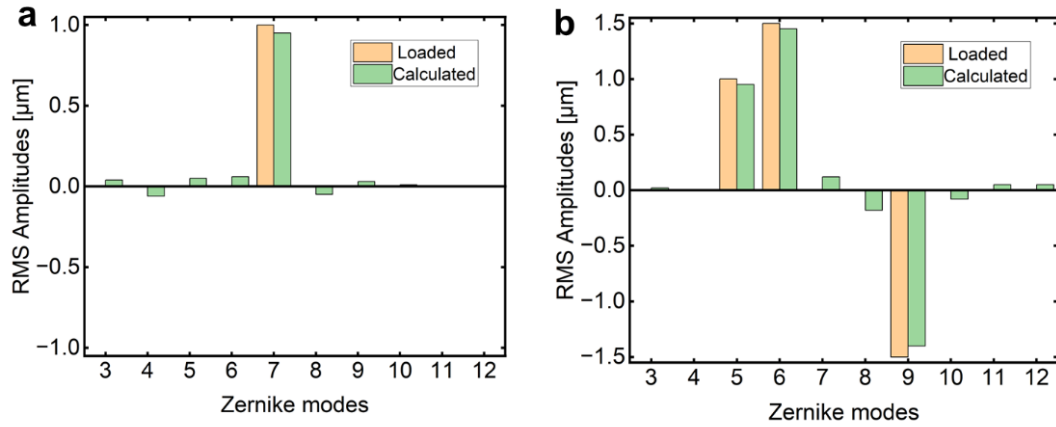


Fig. 2-11 Modal verification of the DM-SHWS transmission matrix. (a) Bar chart comparing commanded (Loaded, orange) and reconstructed (Calculated, green) RMS amplitudes for a single Zernike mode (mode 7). **(b)** Bar chart comparing commanded and reconstructed RMS amplitudes for a superposition of multiple Zernike modes (Modes 3, 6 and 9).

The single-actuator poke test was conducted by incrementally applying a small voltage to each actuator individually and recording the resulting spot shifts. For one representative actuator, the loaded voltage step produced a corresponding phase pattern shown as the blue trace, while the phase pattern reconstructed from measured slopes via the inverse transmission matrix is illustrated by the orange trace, as plotted in Fig. 2-12a. Similar comparisons performed across all 97 actuators are summarized as vertical bar pairs, each indicating the loaded and reconstructed phases for an individual actuator, as presented in Fig. 2-12b. The results demonstrate that the calculated values closely matched the loaded voltages for both the single actuator case and across all 97 actuators.

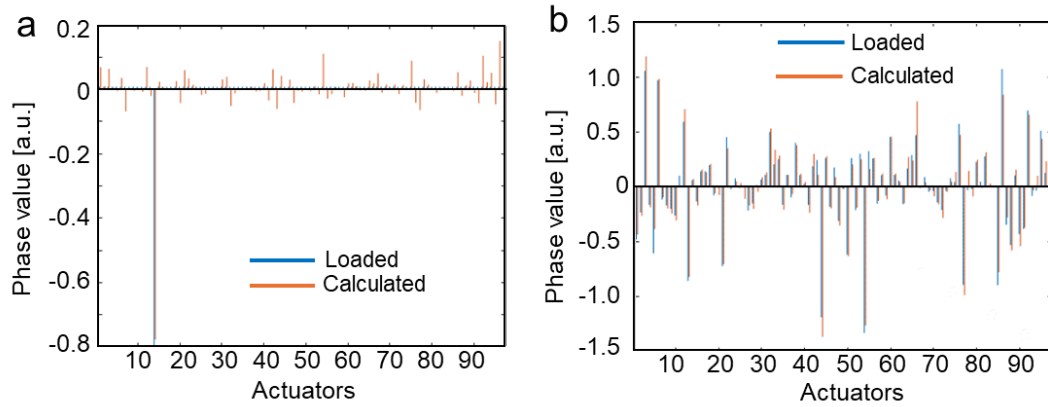


Fig. 2-12 Single-actuator poke verification of the DM-SHWS transmission matrix. (a) Phase profile for one actuator: the known phase deformation (Loaded, blue) and the reconstructed phase from SHWS measurements (Calculated, orange). **(b)** Phase amplitudes for all actuators: each actuator's loaded phase (blue) and calculated phase (orange) plotted in actuator index order.

2.7 AO Correction Results of UCNPs Sample

To demonstrate the performance of the complete adaptive-optics system, we imaged a densely aggregated line of UCNPs, whose aberrations are induced by placing a refractive-index mismatch polydimethylsiloxane. In the absence of AO correction (Fig. 2-13a), the line appears blurred, and its fluorescence intensity is low owing to the aberrated excitation focus. After applying the computed DM flat-map and dynamic aberration correction (Fig. 2-13b), the line becomes sharply resolved and markedly brighter.

The corrected wavefront pattern that the DM imposes is shown at the top right. A transverse intensity profile extracted along the red and blue lines in panels b and a, respectively, is plotted in the lower right inset. The orange curve (AO on) exhibits a pronounced peak and higher baseline than the blue curve (AO off), confirming that the calibrated DM-SHWS transmission matrix successfully restores a diffraction-limited focus even through highly scattering UCNP aggregates.

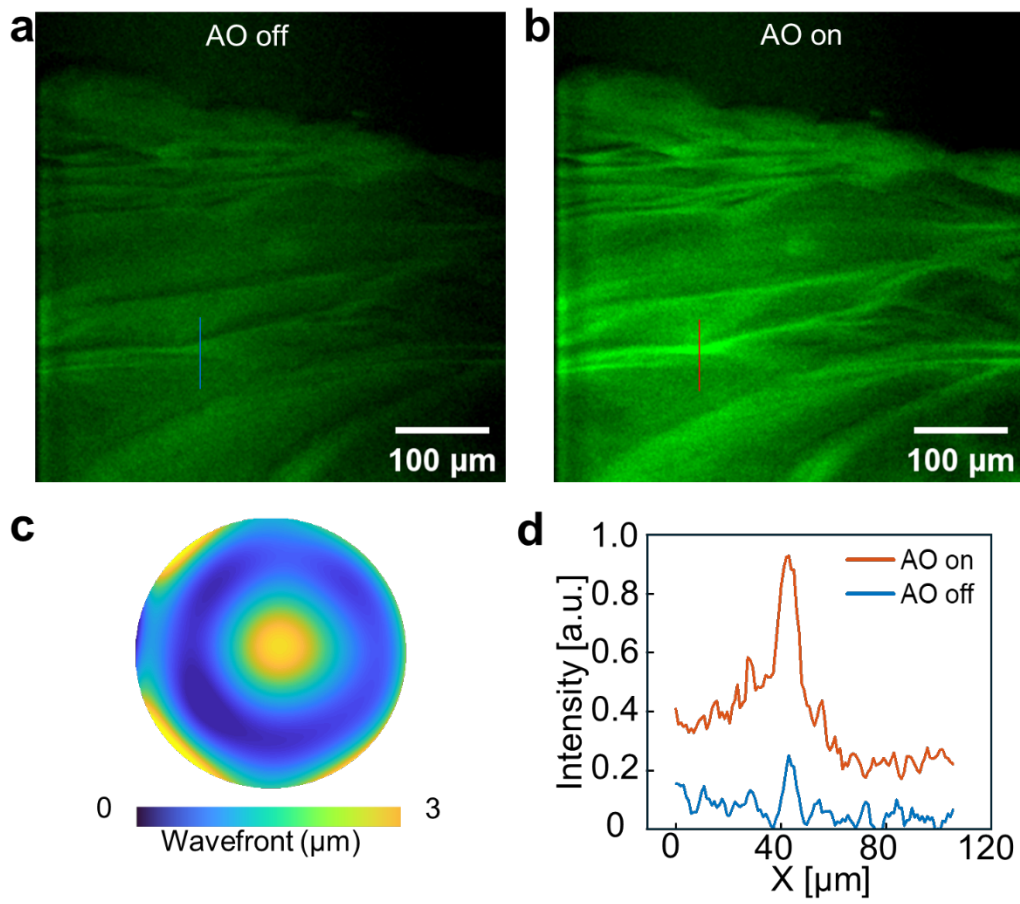


Fig. 2-13 AO-corrected UCNP imaging of a dense UCNP line. (a) Fluorescence image acquired with AO disabled. The blue vertical bar indicates the scan line used for intensity profiling. (b) Fluorescence image acquired with AO enabled. The red vertical bar indicates the corresponding profile location. (c) Wavefront map displayed on the DM during AO correction, color-coded in micrometers. (d) Normalized intensity profiles extracted along the marked lines in (a) and (b).

2.8 Discussion

In this chapter, we have demonstrated a comprehensive implementation of direct wavefront sensing AO in UCNPs-based NLSM. By integrating a DM within the excitation path and employing a customized SHWS to measure the aberrated wavefront from an embedded GS, we achieve real-time compensation of both system and sample aberrations. Our calibration procedures, including DM flat-map generation, transmission-matrix $\mathbf{H}$ measurement via actuator pokes, and modal and single-actuator verification, ensure that the transmission matrix accurately captures the complex

coupling between mirror commands and sensor measurements.

The resulting closed-loop system reproduces diffraction-limited PSFs for large-aberrations sample where uncorrected imaging suffers severe resolution and intensity loss. In particular, imaging of a densely aggregated UCNP line highlights the gains in focal sharpness and signal strength afforded by AO correction. Seamless integration with the ScanImage platform allows wavefront measurement, phase reconstruction, and DM update to occur within standard frame intervals, supporting high-speed functional imaging in scattering media.

The direct AO approach offers several key advantages than indirect AO. It bypasses iterative image-based optimization, eliminating exposure overhead and reducing photodamage. The SHWS-based measurement provides unambiguous, quantitative wavefront maps that drive the DM with high fidelity. Moreover, the ability to pre-flatten the DM surface and isolate dynamic aberration correction maximizes the DM surface's usable stroke and maintains linear response.

The methodologies and results presented in this section establish the essential framework for the subsequent development of a homologous dual-emission upconversion adaptive-optics microscopy, as detailed in Chapter 3.

3 HOMOLOGOUS DUAL-EMISSION UPCONVERSION

ADAPTIVE OPTICS MICROSCOPY

This chapter is reproduced with some adaptations according to a published journal paper: Yao J, Yu Z, Gao Y, et al. *Deep-Penetrating and High-Resolution Continuous-Wave Nonlinear Microscopy Based on Homologous Dual-Emission Upconversion Adaptive Optics*. *Nano Letters* 25(13): 5485-5492 (2025).

3.1 Introduction

Nonlinear optical microscopy (NLOM), exemplified by MPM [34,35,110-112], utilizes nonlinear light-matter interactions to generate fluorescence for imaging. Over the past 20 years, NLOM has been widely used to reveal cellular structures [113,114], biomolecular distributions [115], and the dynamics of life processes[116-119]. In these applications, imaging resolution usually increases with the order of nonlinearity[120], which, however, is practically constrained as conventional nonlinear fluorophores exhibit low-order nonlinearities due to their small absorption cross-section. Consequently, high-intensity ultrafast femtosecond laser pulses are indispensable for generating sufficient nonlinear signals [121]. Additionally, some issues such as photobleaching, phototoxicity [122], and re-excitation background [123] associated with the use of these nonlinear fluorophores cannot be ignored.

UCNPs, typically doped with ytterbium sensitizer ions (Yb^{3+}), have been recently employed as novel probes in NLOM [93,94,98,124-127]. Compared with traditional nonlinear fluorophores, UCNPs possess longer lifetime of the energy levels [128,129], enabling a sequential photon absorption process that converts high-energy NIR excitation into multiple anti-Stokes emissions [130-132]. This bypasses the aforementioned nonlinearity excitation requirements, allowing for the use of

more cost-effective and readily available CW NIR lasers instead of high-intensity femtosecond lasers. Furthermore, UCNPs inherently exhibit higher-order nonlinearity, providing higher resolution and better signal-to-noise ratio (SNR) than traditional nonlinear fluorophores [36,133,134].

Despite these advantages of UCNPs and the use of NIR laser excitation to mitigate scattering, aberrations still persist in deep tissue [97], similar to those encountered in conventional MPM [135]. In deep tissue imaging, optical aberrations and scattering disrupt the formation of a diffraction-limited focus, thereby diminishing signal integrity, contrast, and resolution [136-140]. Traditionally, a direct-wavefront-sensing AO method [38,108,141,142] has been developed to recover to the diffraction-limit focus by correcting aberrations along the excitation path in scattering media. This method employs a SHWS to measure wavefront distortion by creating a fluorescent GS inside the specimen. The clarity of the GS image across SHWS elements is essential for effective aberrations correction [143]. However, in tissues such as the mammalian brain, strong scattering attenuates the GS-forming ballistic fluorescence and generates a diffused background that can obscure the ballistic signal, complicating aberration measurement. Optical scattering is wavelength-dependent, with shorter wavelengths experiencing more scattering. Consequently, while the visible (VIS) GS of the UCNPs offers improved imaging quality due to their high-order nonlinearity, it is not optimal for aberration measurement in deep tissues because of its susceptibility to scattering.

In this chapter, we proposed a deep-penetrating and high-resolution CW nonlinear microscopy based on homologous dual-emission upconversion AO (H DU-AO) by employing homologous dual-emission feature of the Tm^{3+} and Yb^{3+} co-doped UCNPs upon CW excitation at 975 nm. The NIR (800 nm) emission of UCNPs serves as the GS due to its reduced scattering and its wavefront closer alignment with the excitation light's distortion profile, and the VIS (455 nm) emission is utilized for high-resolution imaging owing to its four-photon upconverting effect. Through comparison it reveals

that the 455 nm emission offers superior resolution and SNR, whereas the 800 nm emission provides the capability to penetrate more deeply within the mouse brain environment. Subsequently, we demonstrated the applicability of the proposed method to in vitro imaging, clearly resolving nanoparticles with a lateral resolution of 480 nm within a complex 500 μm -thick mouse brain environment by integrating our approach into a home-built nonlinear laser scanning microscope.

3.2 Methods and Materials

3.2.1 Principle of HDU-AO

The detailed procedure of HDU-AO is illustrated in Fig. 3-1. The UCNPs, co-doped with Tm^{3+} and Yb^{3+} , are designed to emit two distinct upconverted wavelengths: VIS at 455 nm and NIR at 800 nm, upon excitation with a 975 nm CW laser. As shown in Fig. 1a, the VIS emission exhibits a much higher nonlinear effect compared with the NIR emission, resulting in a shaper PSF, which is chosen for imaging. Conversely, the NIR emission is used as the GS for aberrations correction because it experiences less scattering and provides a significantly closer alignment with the distortion profile of the excitation light than the VIS emission. This results in a much clearer and brighter image consisting of spot matrix captured by the SHWS (Fig. 3-1b). By leveraging the 800 nm emission as the GS for correction in deeper tissues, we can achieve high-resolution AO imaging using the 455 nm emission even at challenging depths (Fig. 3-1c).

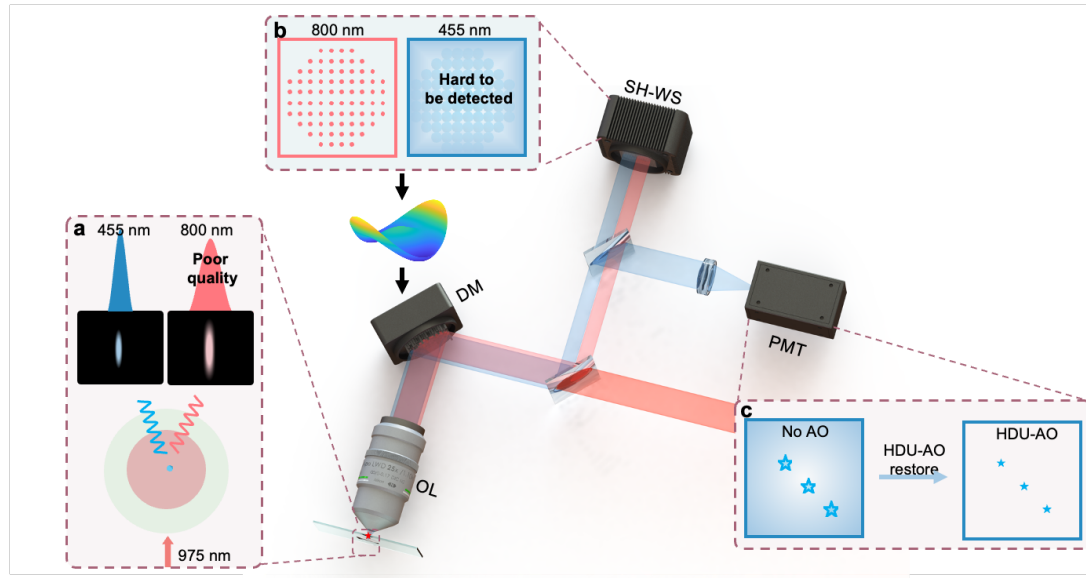


Fig. 3-1 Principle of the proposed HDU-AO microscopy. (a) The emission characteristic of Tm^{3+} and Yb^{3+} co-doped UCNPs. (b) Illustrative spot diagrams captured by the SHWS. (c) Illustrative images captured by the photomultiplier tube (PMT).

3.2.2 Optical Setup

The experiment setup of HDU-AO includes a nonlinear imaging module, an SHWS, and a wavefront correction device (Fig. 3-2). Briefly, the excitation light (975 nm) from a laser (OS8147-975-900, B&A technology Co., Ltd, and Chameleon Ultra, Coherent) was expanded ten folds by a lens pair (L1: AC254-30-B, L2: AC254-300-B, Thorlabs) to overfill the aperture of a deformable mirror (DM, DM140A-35-P01, Boston Micromachine). After the DM, the excitation beam was raster scanned by a pair of galvanometer mirrors (Galvo XY, TS8203, Sunny Technology) and then focused onto the samples by a water-immersion objective (N16XLWD-PF Nikon, 25XAPO-MP Nikon). Note that the surfaces of the DM, Galvo X&Y, and the back pupil of the objective were made mutually conjugated through three pairs of relay lenses operating in the 4f configuration (L3: AC254-300-B, L4: AC254-250-B; L5: AC508-080-AB and AC508-080-AB lenses coupled; L6: two AC508-080-AB lenses coupled, Thorlabs). For nonlinear imaging, the fluorescence was collected by the objective and then reflected by a dichroic mirror DMBS (T715LP, Chroma) and focused by another lens pair (L7: LA1145-A, L8: LA1805-A, Thorlabs) onto a photomultiplier tube (PMT,

H7422P-40, Hamamatsu). A bandpass filter (MV450/20 nm, Chroma) was placed before the PMT to purify the fluorescence detection.

For aberration correction, the NIR fluorescence from the GS was descanned by Galvos X and Y, separated from the excitation light by a dichroic mirror BS2 (FF875-Di01-25x36, Semrock), passed through a beam expander in 4-f configuration (AC254-100-AB, AC254-200-AB, Thorlabs), purified by a filter wheel (FWM12S, LBTEK), and the fluorescence filter was switchable between VIS BS3 (FF01-720/SP-25, Semrock) and NIR filter (FF01-792/64-25, Semrock), and finally detected by the SHWS. The SHWS consisted of a microlens array (#64–483, Edmund Optics) conjugated with the objective rear pupil and a camera (Dhyana 400BSI, Tucsen) placed at the focal plane of the microlens array.

Besides, to compare the imaging performance of the 455 nm and 800 nm emissions, we split the light directed to the SHWS into two paths by a beam splitter BS₃ (BS2555-T3M, JCOPTIX): one to the SHWS and the other to a NIR-specific PMT detector (H7422P-40, Hamamatsu, peak sensitivity wavelength: 800 nm) equipped with a filter (FF01-792/64-25, Semrock). It should also be noted that BS1 was removed from the light path through a switch (ELL6K, Thorlabs) when measuring the penetration depth of the 455 nm emission.

A customized MATLAB code based on the ScanImage program[144] was used to capture the nonlinear excitation images, measure and compensate for the optical aberration, and control the devices. The SHWS and the DM were synchronized via a MATLAB program. A National Instruments DAQ card (PCI6110) was used to control and synchronize the system as well as digitize the obtained fluorescence signals.

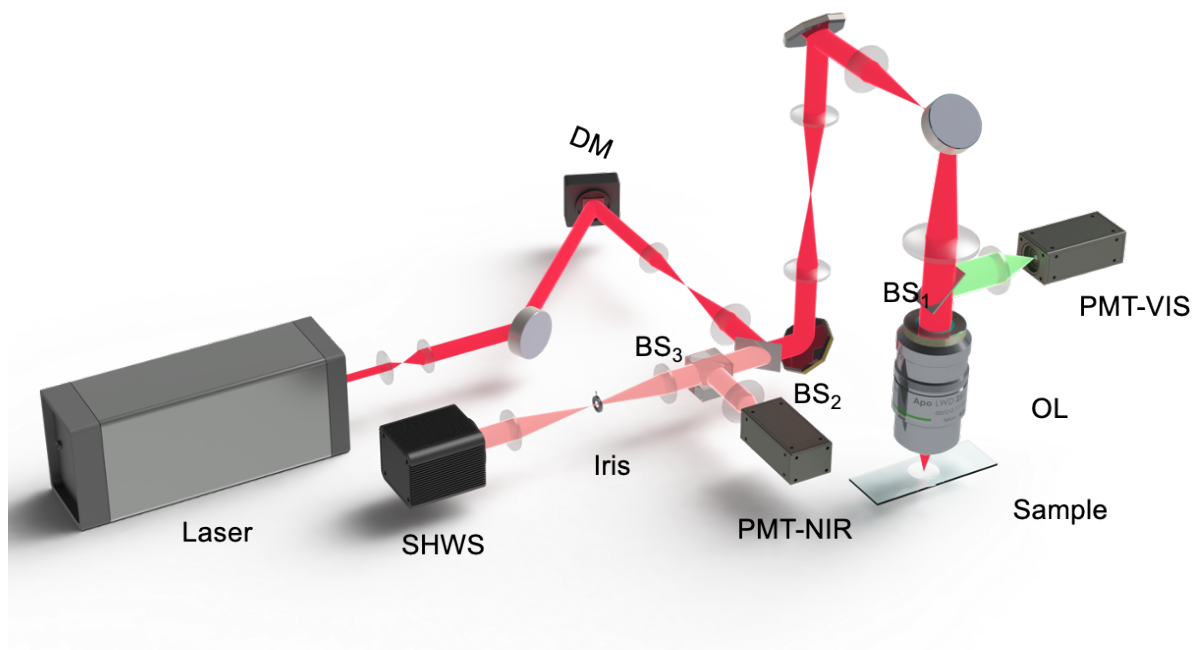


Fig. 3-2 The optical setup of proposed method. BE: beam expander; M: mirror; DM: deformable mirror; L: lens; BS: beam splitter; X-S: x-scanner; Y-S: y-scanner; PMT: Photomultiplier tube; OL: objective lens.

3.2.3 Synthesis of UCNPs

Purchase of materials. Sodium hydroxide (NaOH, methanol solution, $\geq 99.9\%$), ammonium fluoride (NH_4F , methanol solution, $\geq 99.9\%$), oleic acid (OA, analytical reagent (AR)) and 1-octadecene (ODE, $\geq 90\%$ (GC)) were purchased from Aladdin. Yttrium (Y, acetate hydrate, 99.9%), thulium (Tm, acetate hydrate, 99.9%), ytterbium (Yb, acetate hydrate, 99.9%) were purchased from Sigma-Aldrich. Methanol (reagent grade), ethanol (reagent grade) and cyclohexane (reagent grade) were purchased from Sinopharm Chemical Reagent.

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Synthesis of NaYF₄:Yb/Tm (50/4%) core nanoparticles. The designed lanthanide-doped upconversion nanoparticles were synthesized based on a protocol reported previously with some modifications[131]. Initially, added a 5 mL portion of an aqueous solution of Ln(CH₃CO₂)₃, containing 0.04 mmol Tm³⁺, 0.46 mmol Y³⁺, and 0.5 mmol Yb³⁺, to a 100 mL round-bottom flask that already held 7.5 mL OA and 17.5 mL ODE. The stirring mixture was heated to 120°C for 10 minutes to eliminate water from the solution, then continued heating to 150°C for 40 minutes to form lanthanide-oleate precursor. After naturally cooling to 40°C, 10 mL NH₄F (0.4 mmol/mL in methanol) and 2.5 mL of NaOH (1 mmol/mL in methanol) were added into the flask. Next, stirred the mixture at 40°C for at least two hours to clarify it, then heated the solution under vacuum to 100°C for 30 minutes to eliminate methanol, followed by filling the flask with argon gas. The mixture was heated to 300°C for 1.5 hours under anhydrous and anaerobic conditions, then cooled down to the room temperature. To precipitate nanoparticles, added 15 mL anhydrous ethanol to the mixture, and then collect the nanoparticles by centrifuging at 7500 r.p.m. (relative centrifugal force (RCF) = 4788×g) for 5 minutes. The obtained nanoparticles were washed several times with anhydrous ethanol and cyclohexane, and finally re-dispersed them into 8 mL cyclohexane for subsequent use.

Synthesis of NaYF₄:Yb/Tm@NaYF₄ core-shell nanoparticles. The synthesis of the NaYF₄ shell is analogous to that of the NaYF₄:Yb/Tm (50/4%) core[145]. A 1 mmol solution of Y(CH₃CO₂)₃ (0.2M), 7.5 mL OA, and 17.5 mL ODE was put into a 100 mL three-necked flask. Then heated the mixture to 150°C with stirring for 50 minutes. Subsequently, upon cooling the flask to 90°C, 4 mL of the prepared NaYF₄:Yb/Tm (50/4%) core was injected into the flask. The subsequent steps were identical to those for the synthesis of the NaYF₄:Yb/Tm (50/4%) core, except that the core-shell nanoparticles were ultimately stored in 4 mL cyclohexane.

3.2.4 Tissue Sample Preparation

BALB/c mice aged 8-12 weeks were used for the experiment. All experiments were performed in compliance with the protocols approved by the Guangdong Provincial

Animal Care and Use Committee and following the guidelines of the Animal Experimentation Ethics Committee of Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences. Mice were first anesthetized using an intraperitoneal injection of a ketamine-xylazine mixture to ensure deep sedation. Following anesthesia, the mice were euthanized through cervical dislocation as per ethical standards. The brain was immediately extracted from the skull using fine dissection tools, taking care not to damage the tissue. The extracted brain was then rinsed in cold phosphate-buffered saline to remove any remaining blood. Once extracted, the brain was embedded in Optimal Cutting Temperature compound to prepare for cryosectioning. The embedding step involved positioning the brain in the desired orientation for sectioning, followed by freezing the brain block using dry ice or storing it at -80°C until the tissue was fully frozen. Once the brain was fully solidified, it was placed in a cryostat, which was pre-cooled to a temperature between -20°C to -30°C to ensure optimal cutting conditions.

The frozen brain tissue was then sectioned into slices of varying thickness using the cryostat. Brain slices of 100 μm , 200 μm , 300 μm , 400 μm , 500 μm , and 600 μm were prepared, as depicted in Figure S2. Each slice was carefully collected and transferred onto pre-labeled glass slides to preserve the integrity of the brain structure. The slides were stored at -20°C or colder until they were ready for further analysis.

3.3 Results and Discussion

3.3.1 Characterization of UCNPs

The UCNPs utilized in this study are co-doped with Tm^{3+} and Yb^{3+} , exhibiting the remarkable property of emitting dual emissions at both 455 and 800 nm when excited by a 975 nm CW laser. These particles are characterized by uniform morphologies with an average size of 33 nm, as evidenced by the transmission electron microscopy (TEM) image presented in Fig. 3-3a. As depicted in Fig. 3-3b, when excited by a 975 nm CW laser, the Yb^{3+} and Tm^{3+} ions successively absorb multiple photons, transitioning from the ground state $^2\text{F}_{7/2}$ to the excited states $^1\text{D}_2$ and $^3\text{H}_4$, subsequently reaching the $^3\text{F}_4$

and $^3\text{H}_6$ states, which are responsible for the emissions at 455 and 800 nm, respectively. In the proposed HDU-AO, we aim to utilize both the 455 nm and 800 nm emissions effectively. As illustrated in Fig. 3-1, by pre-compensating the aberrations through the 800 nm emission wavefront sensing, an aberration-free focus is capable of exciting the 455 nm fluorescence signals, which can then be detected by a PMT. Through our experiments, we have identified an optimal concentration of Tm^{3+} doping. This specific concentration allows us to balance the intensities of both the 455 nm and 800 nm emissions, ensuring their effective use in HDU-AO (Fig. 3-3c). Figure 3-3d shows the nonlinear optical response behaviors of UCNPs. The results demonstrate that the emission at 800 nm corresponds to a 2P process ($\text{Slope}_{800} = 1.69$), while the 455 nm emission results from a four-photon excitation process ($\text{Slope}_{455} = 4.03$). The 800 nm emission demonstrates superior excitation efficiency over the 455 nm emission at lower power densities, enabling effective laser excitation and subsequent detection via SHWS under less strict excitation conditions.

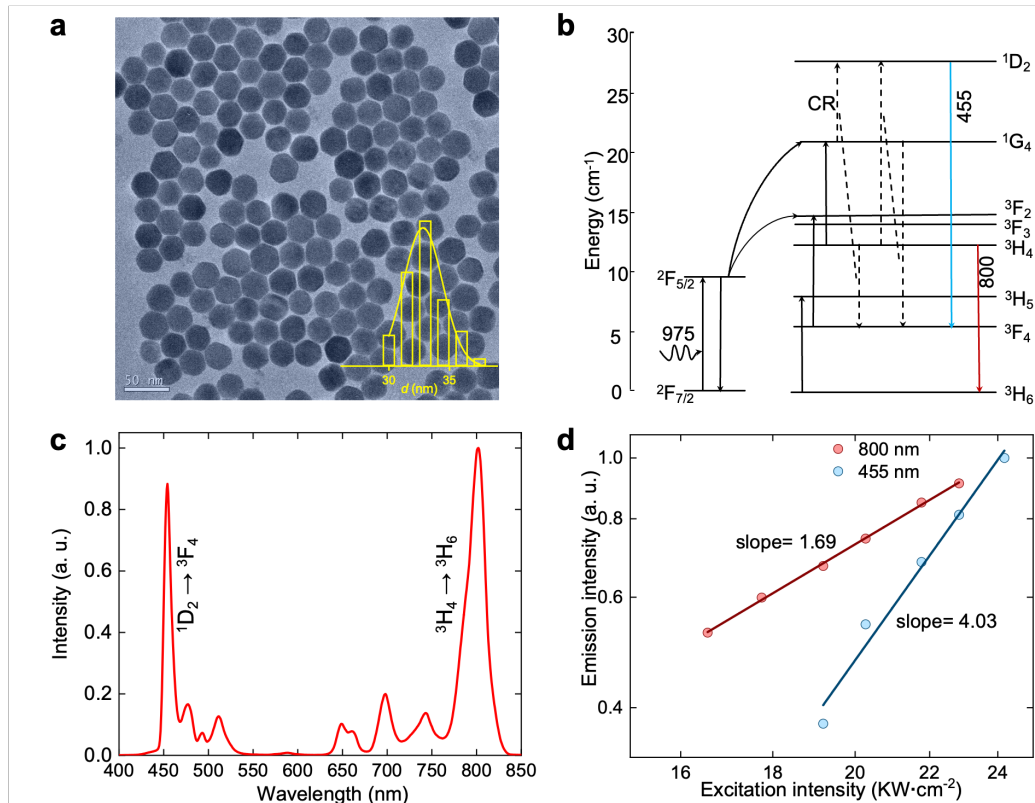


Fig. 3-3 Characteristics of the utilized UCNPs co-doped with Tm^{3+} and Yb^{3+} . (a) TEM

images of utilized UCNPs, whose average size is around 33 nm. Scalebar, 50 nm. **(b)** The energy diagram and upconversion process of Yb^{3+} and Tm^{3+} co-doped UCNPs. The sensitizer Yb^{3+} ions initiates the photon upconversion process by the 975 nm excitation. The NIR upconversion emissions mainly composed of the two-photon excited state (800 nm, $^3\text{H}_4 \rightarrow ^3\text{H}_6$) and four-photon excited state (455 nm, $^1\text{D}_2 \rightarrow ^3\text{F}_4$). **(c)** The upconversion emission spectrum of UCNPs under 975 nm excitation. **(d)** The excitation intensity-dependent emission curve, $\text{Slope}_{455} = 4.03$, $\text{Slope}_{800} = 1.69$.

3.3.2 Imaging Comparison with the VIS and NIR Emissions

A comparative analysis of the imaging quality and penetration capability of the VIS and NIR emissions is conducted, as shown in Fig. 3-4. The scanning microscopic images of the UCNPs (without a scattering cover), using VIS and NIR emission, are presented in Fig. 3-4a. The results of the 800 nm emission exhibit significant background noise when compared to those of the 455 nm emission, as shown in Fig. 3-4a. This is primarily due to the 800 nm emission being associated with a two-photon excitation process ($\text{Slope}_{800} = 1.69$, Fig. 3-3d), which leads to a considerable out-of-focus fluorescence background. Furthermore, the resolutions of the two UC beads presented in Fig. 3-4a(iii) and (iv) show a notable improvement, with the resolutions enhancing from 990 nm and 850 nm to 610 nm and 590 nm (Fig. 3-4b), respectively. These results indicate that the VIS emission provides a substantially higher SNR and superior resolving power (Fig. 3-4a), suggesting that the VIS emission is more suitable for high-resolution imaging.

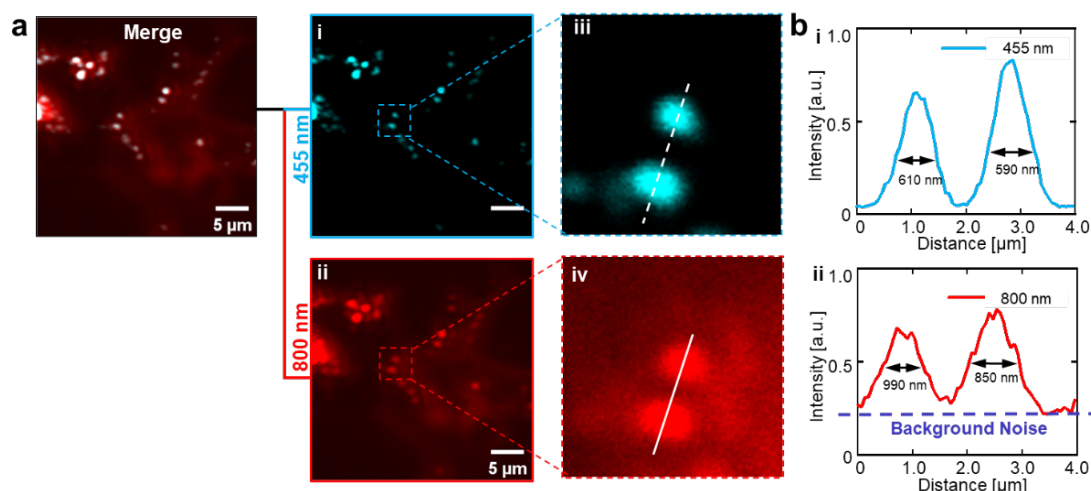


Fig. 3-4 Comparison of imaging quality with the 455 nm (VIS) and 800 nm (NIR) emissions when there is no scattering cover above the UCNPs. (a) Comparison of imaging quality. (b) Corresponding profiles.

To assess the penetration capability, the UCNP sample is covered with various mouse brain slices from BALB/c mice (Fig. 3-5a), with thickness ranging from 100 μm to 600 μm in increments of 100 μm (Fig. 3-5b). As the thickness of the brain slice increases, images captured by the SHWS using VIS and NIR emissions are shown in Figs. 3-6a and 3-6b, respectively. The results demonstrate that the VIS emission's spot diagrams become blurred and indistinguishable, particularly at thickness beyond 300 μm, while the spot diagrams of the NIR emission remain clearly distinguishable all through the experiments. This indicates that the NIR emission is more appropriate for use as the GS. Because VIS and NIR emissions are homologous, HDU-AO provides a robust and straightforward strategy to achieve high-resolution imaging in deep tissue under CW excitation.

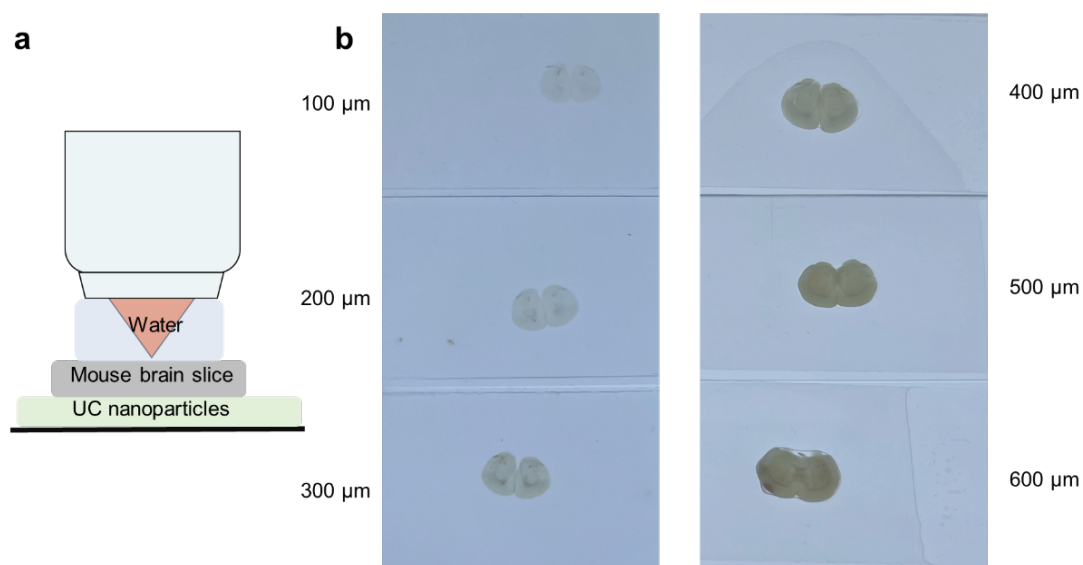


Fig. 3-5 Depiction of the samples for comparison of penetration capability. (a) Sample configuration. (b) mouse brain slices of various thickness to cover the UCNPs.

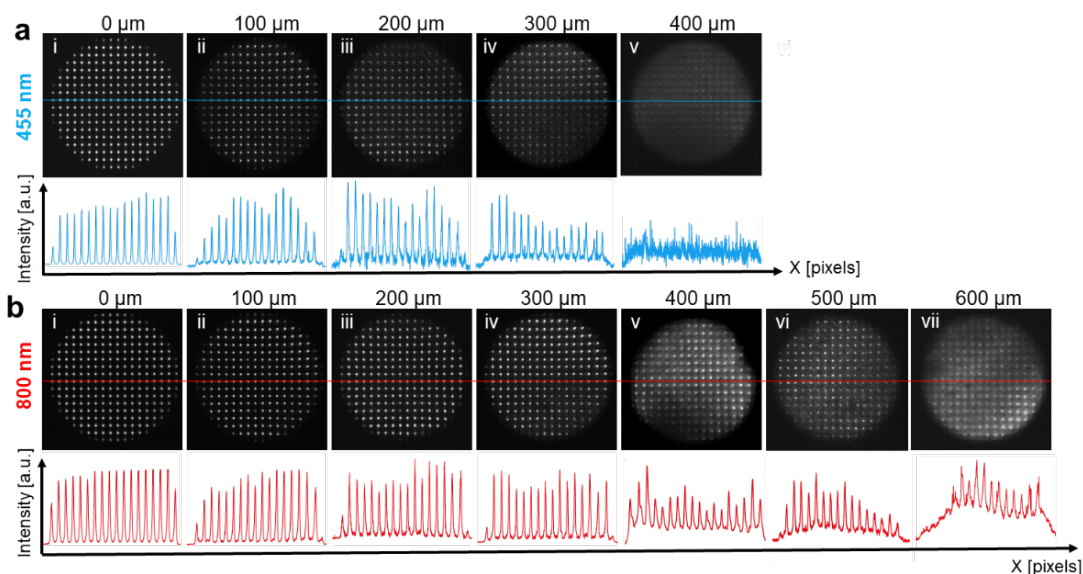


Fig. 3-6 Spot diagrams captured by the SHWS through mouse brain slices of different thickness for 455 nm (a) and 800 nm (b) emissions, respectively. The blue curves in (a) and the red curves in (b) are the representative horizontal profiles of the corresponding spot diagram.

3.3.3 AO Correction of Aberrations

The aberration from the refractive mismatch is first investigated using the proposed method. Cuboid Polydimethylsiloxane (PDMS) pieces (Refractive index:1.41) are positioned between the objective lens and the sample, resulting in significant refractive mismatch (Fig. 3-7a). The SHWS spot pattern recorded for this PDMS type (Fig. 3-7c) shows radially symmetric displacements, and Zernike-polynomial reconstruction[146].of the corresponding spot shifts (Fig. 3-7d) confirms that primary spherical aberrations dominate the wavefront error. Under $\sim 25 \text{ kW cm}^{-2}$ excitation, the ensemble of UCNPs images before and after HDU-AO correction (Fig. 3-7b) exhibits a marked increase in sharpness: blurred, low-contrast particles resolve into well-defined spots. Focusing on a single nanoparticle (Fig. 3-8, boxed in Fig. 3-7b), the lateral and axial intensity profiles (Figs. 3-8b and 3-8b) reveal a roughly twofold reduction in full-width at half-maximum and a comparable increase in peak brightness after aberration correction.

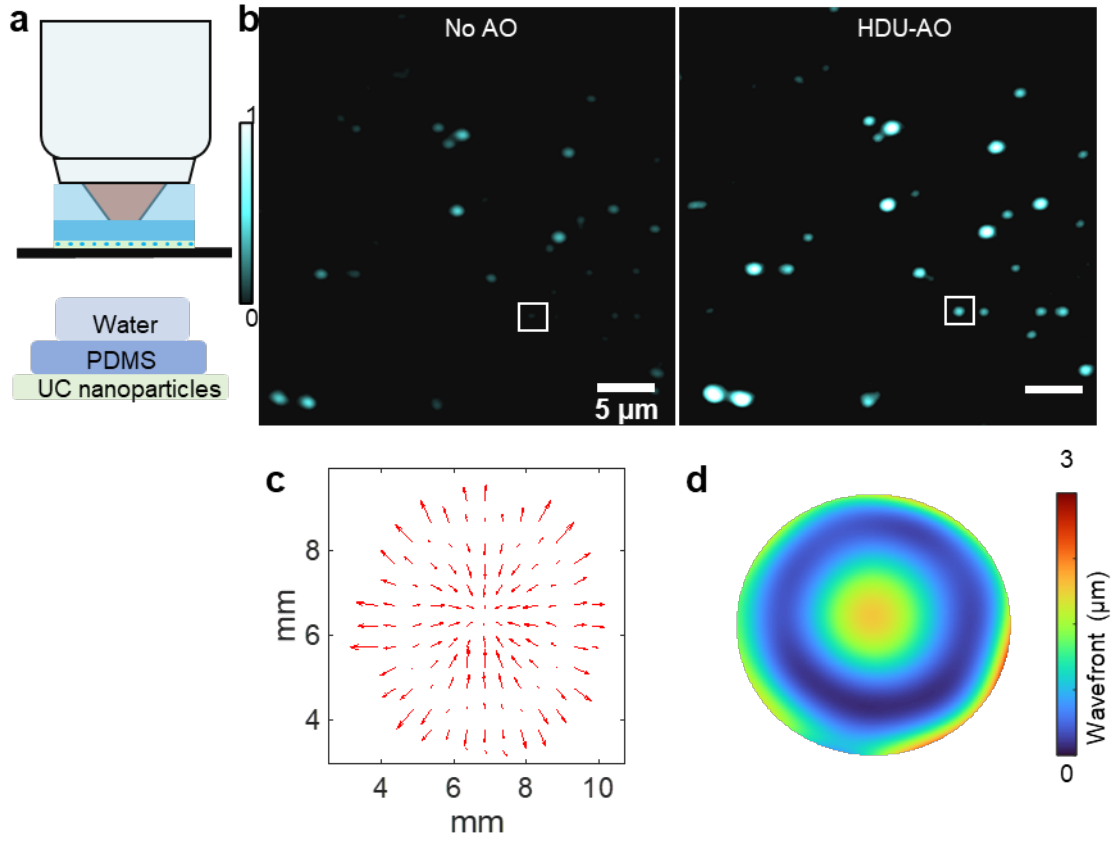


Fig. 3-7 HDU-AO compensation for aberrations induced by refractive mismatch of the cuboid PDMS. (a) Sample configurations for cuboid PDMS. **(b)** Max intensity project (MIP) image comparisons of aberrations corrections without and with AO. **(c)** Spot shift diagrams corresponding to the reference aberration-free pattern. **(d)** Reconstructed wavefront patterns based on the Zernike modes. The scale bars in this figure represent 5 μm in large images and 500 nm in zoom-in images, respectively.

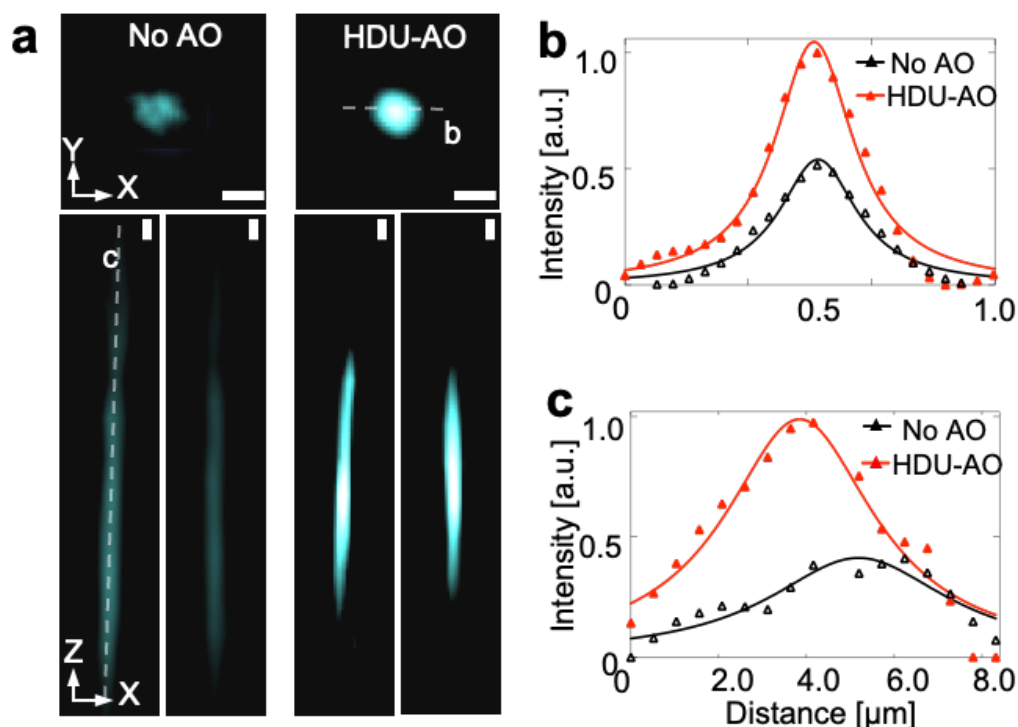


Fig. 3-8 One-bead images for cuboid PDMS HDU-AO imaging. (a) Three views of a single bead image extracted from the whole-FOV images, scale bar: 1 μm . (b, c) Intensity profile comparisons along the indicated lines in (a).

Next, we tilted the oblique PDMS pieces between the objective lens and the sample, resulting in significant another types of aberrations (Figs. 3-9a). The spot diagrams captured by the SHWS for oblique PDMS type (Figs. 3-9c) are analyzed, and the aberrations are subsequently reconstructed using spot-shift diagrams and Zernike polynomials (Figs. 3-9d). The calculated results reveal that the oblique configuration introduces substantial coma and astigmatism (Fig. 3-9d). After correction, a significant enhancement in image sharpness and clarity for nanoparticles is also observed (Figs. 3-9 and 3-10). Notably, nanoparticles with sharp deformation due to asymmetrical aberrations regain their normal, circular shape after correction (Fig. 3-10a). To demonstrate the efficacy more concretely, one-bead images from three different perspectives (Figs. 3-10a) are extracted from the full field of view (FOV). As seen, after correction, unwanted ghost signals are effectively eliminated, resulting in significant improvement in both lateral and axial resolution, as well as the peak signal intensity, as

depicted in Figs. 3-10b and 3-10c which show the lateral (b) and axial (c) intensity profiles for each oblique type respectively.

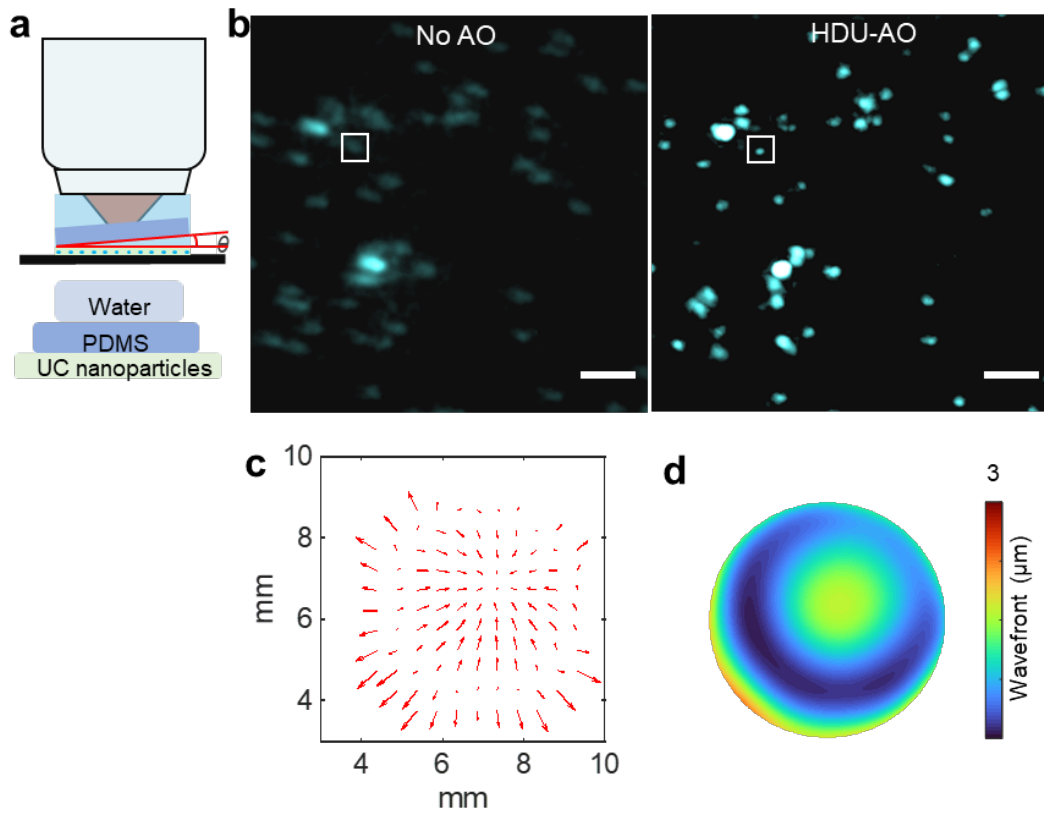


Fig. 3-9 HDU-AO compensation for aberrations induced by refractive mismatch of the oblique PDMS. (a) Sample configurations for oblique PDMS. **(b)** MIP image comparisons of aberrations corrections without and with AO. **(c)** Spot shift diagrams corresponding to the reference aberration-free pattern. **(d)** Reconstructed wavefront patterns based on the Zernike modes. The scale bars in this figure represent 5 μm in large images and 500 nm in zoom-in images, respectively.

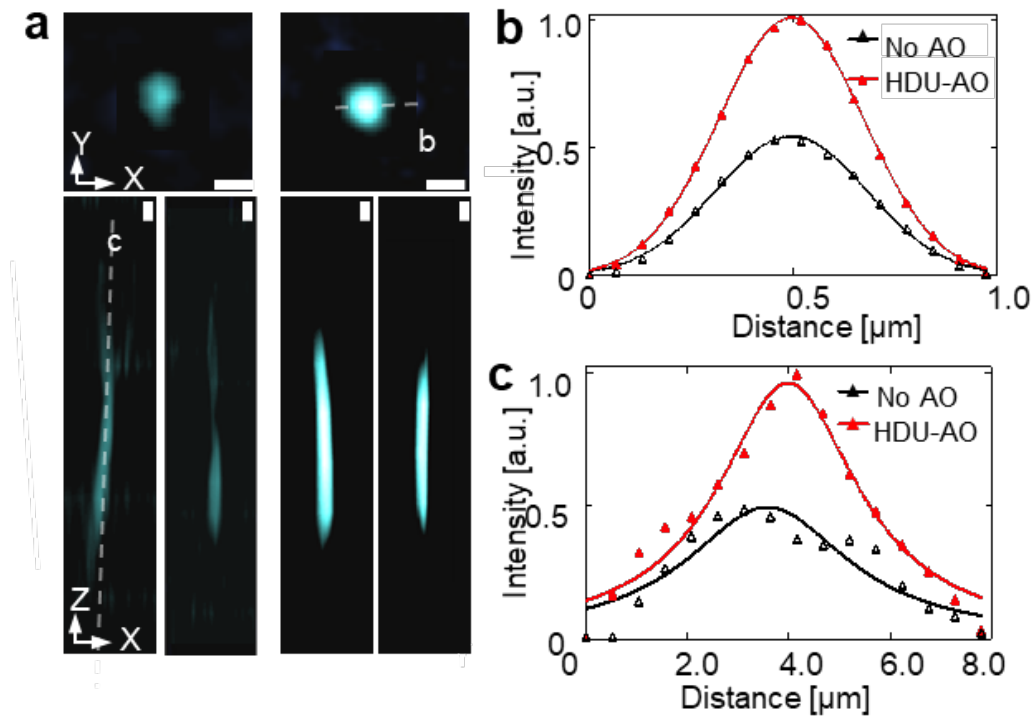


Fig. 3-10 One-bead images for oblique PDMS HDU-AO imaging. (a) Three views of a single bead image extracted from the whole-FOV images, scale bar: 1 μm . (b, c) Intensity profile comparisons along the indicated lines in (a).

These findings demonstrate that the HDU-AO framework can reliably correct both isotropic spherical aberrations and complex, directionally biased distortions introduced by refractive-index mismatches. By employing a bespoke SHWS to precisely measure the aberrated wavefront and applying the corresponding DM corrections, the system consistently restores near-diffraction-limited imaging of upconversion nanoparticles under a variety of sample-mounting conditions. This capability establishes a robust platform for high-fidelity, deep-tissue nanoscopy in optically demanding environments.

3.3.4 Deep-tissue Imaging with HDU-AOM

To evaluate the performance of our HDU-AO method in a deep-tissue imaging scenario, we conducted fluorescence imaging through a 500- μm thick fixed mouse brain slice derived from BALB/c mice, which serves as a representative complex biological scattering medium (Fig. 3-11a). Due to the heterogeneous refractive indices and scattering properties inherent to brain tissues, the excitation wavefront experiences

significant distortion, leading to severely compromised focusing of the excitation beam. Consequently, without correction, the upconversion fluorescence signals emitted by the nanoparticles beneath the brain tissue were indistinct and buried within background noise, thus rendering them practically invisible (Fig. 3-11b, left). After applying HDU-AO correction, a substantial improvement in the visibility and spatial resolution of the nanoparticles was clearly observed, as demonstrated by the comparative color-coded images in Fig. 3-11b. To quantitatively characterize the aberrations induced by the brain slice, SHWS spot-shift diagrams were analyzed (Fig. 3-11c), and Zernike polynomial decomposition was subsequently applied to reconstruct the wavefront distortion pattern (Fig. 3-11d). These analyses revealed that the predominant aberration introduced by the 500- μm brain slice was spherical aberration, originating primarily from refractive-index mismatches and forward scattering in the tissue. All images were acquired under a moderate excitation power of approximately 60 mW prior to entering the mouse brain slice, ensuring both adequate excitation and minimal photodamage. Collectively, these results confirm the capability of HDU-AO to robustly compensate for significant spherical aberrations in realistic biological imaging conditions, thus enhancing imaging quality at substantial tissue depths.

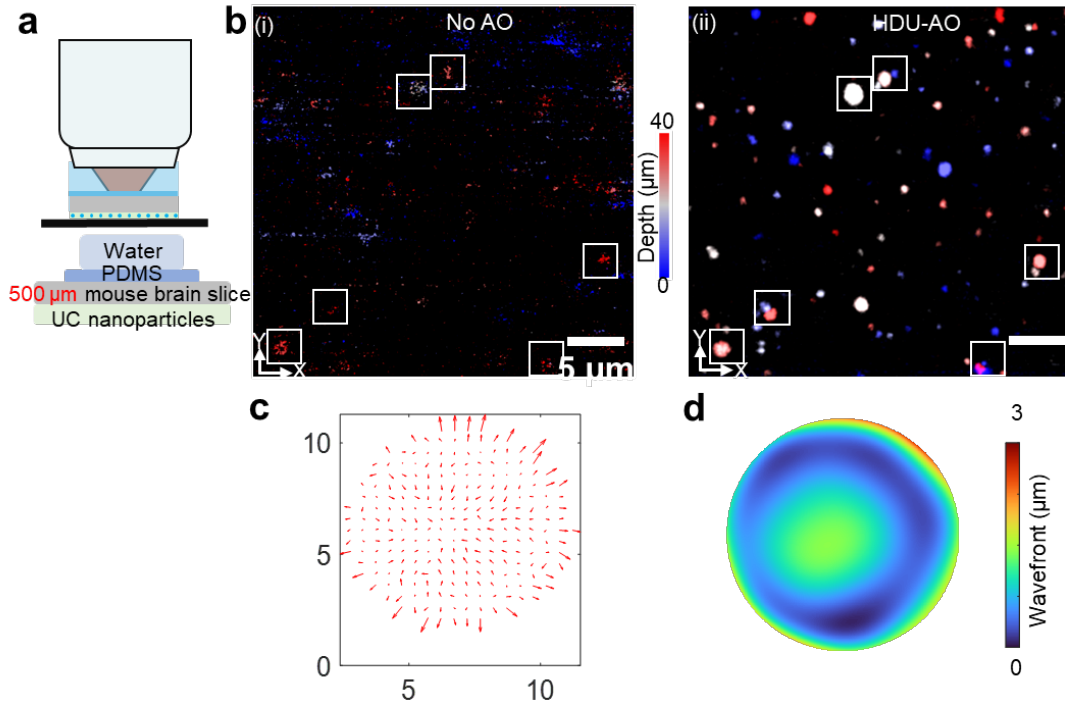


Fig. 3-11 HDU-AO imaging in deep tissue (through mouse brain). (a) Sample configuration. (b) Comparison of $33\ \mu\text{m} \times 33\ \mu\text{m} \times 40\ \mu\text{m}$ volumes of 20 slices with and without HDU-AO, color-coded by depths. (c) Spot shift diagrams corresponding to the reference aberration-free pattern and the wavefront patterns reconstructed by Zernike modes. (d) Reconstructed wavefront patterns based on the Zernike modes. The scale bars in this figure represent $5\ \mu\text{m}$ in large images and $500\ \text{nm}$ in zoom-in images, respectively.

To quantitatively validate the capability of our HDU-AO method in correcting tissue-induced aberrations, we selected representative imaging regions at two different depths beneath the fixed mouse brain slice: $522\ \mu\text{m}$ (Fig. 3-12) and $530\ \mu\text{m}$ (Fig. 3-13). The magnified subregions from both imaging depths clearly illustrate a pronounced improvement in both fluorescence signal intensity and spatial resolution following HDU-AO correction (Figs. 3-12b–c and 3-13b–c). Prior to correction, tissue scattering and refractive-index mismatches substantially distorted the excitation wavefront, resulting in severely aberrated foci at the objective focal plane. Such aberrations compromised the excitation efficiency for VIS emissions, causing larger nanoparticles to exhibit diffuse and irregular morphologies (Figs. 3-12b(i) and 3-13b(i)), and preventing the effective excitation of many smaller particles altogether (Figs. 3-12b(i) and 3-13b(ii)). Consequently, fluorescence intensity was greatly diminished, and spatial

resolution significantly degraded. After applying the HDU-AO correction, however, we observed a dramatic recovery in the excitation efficiency, reflected by a more than five-fold enhancement in the peak fluorescence intensity of nanoparticles (Figs. 3-12d and Figs. 3-13d). Correspondingly, lateral and axial resolutions were significantly improved, as indicated by the narrower intensity profiles and the restoration of symmetrical, sharply defined nanoparticle shapes. Furthermore, Fourier spectral analyses (Figs. 3-12a(iii-iv) and Figs. 3-13a(iii-iv)) reveal that, following HDU-AO correction, previously indistinguishable high-frequency spatial components become clearly discernible, confirming substantial restoration of spatial resolution. Notably, the corrected images exhibit a lateral resolution of approximately 480 nm [Figs. 3-12d(ii)] and 510 nm [Figs. 3-13d(ii)] respectively, approaching the diffraction limit achievable in tissue-free conditions.

Overall, these results unequivocally confirm that the HDU-AO system, using the NIR emission from UCNPs as an intrinsic guide star, can robustly achieve near diffraction-limited VIS fluorescence imaging even under significant tissue scattering conditions. This capability highlights the potential of our method for deep-tissue, high-resolution imaging applications, particularly in complex biological environments where conventional optical methods typically fail.

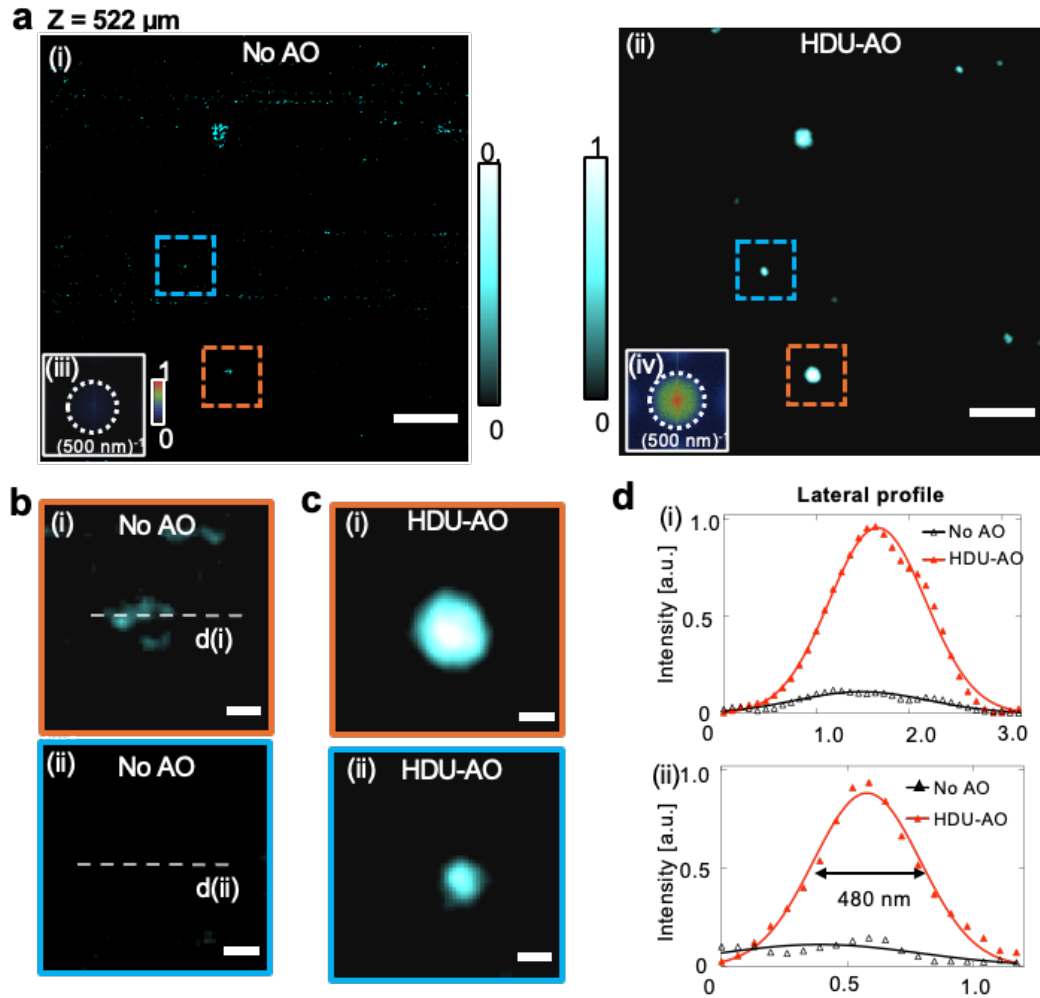


Fig. 3-12 HDU-AO imaging at 522 μm (through mouse brain). (a) Images at 522 μm depths with and without HDU-AO. (b, c) One-bead images extracted from images at 522 μm depths with and without HDU-AO. (d) Intensity profile comparisons along the indicated lines in (b-c). The scale bars in this figure represent 5 μm in large images and 500 nm in zoom-in images, respectively.

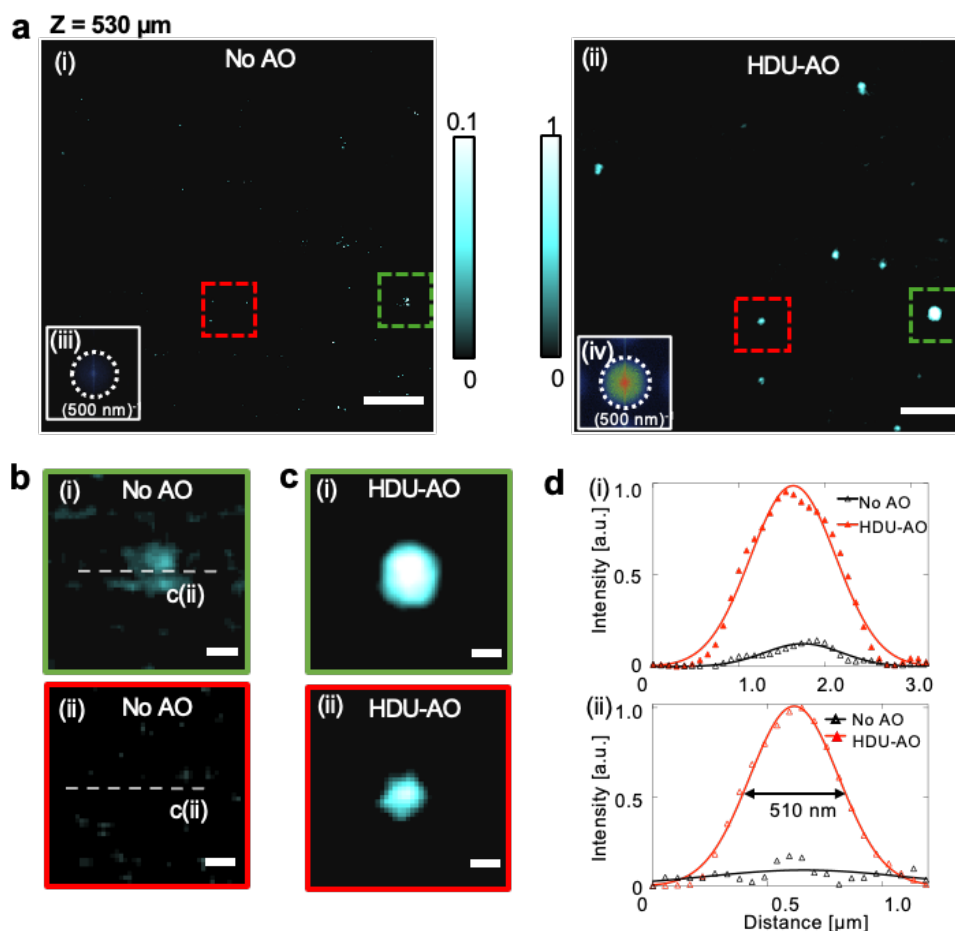


Fig. 3-13 HDU-AO imaging at 530 μm (through mouse brain). (a) Images at 530 μm depths with and without HDU-AO. (b) One-bead images extracted from images at 530 μm depths with and without HDU-AO. (c) Intensity profile comparisons along the indicated lines in (b-c). The scale bars in this figure represent 5 μm in large images and 500 nm in zoom-in images, respectively.

3.4 Summary

In this chapter, we introduced a CW nonlinear imaging microscopy based on HDU-AO, a novel approach to achieve high-resolution in deep tissues that leverages Tm^{3+} and Yb^{3+} co-doped UCNP capable of emitting at both 455 nm and 800 nm simultaneously under a 975-nm CW excitation. This dual-emission feature of UCNP offers a new perspective for measuring and correcting aberration to achieve high resolution imaging in deep tissue. By harnessing the homologous dual-emission feature and other advantages of UCNP, this innovative configuration effectively overcomes traditional

AO limitations in deep tissues, achieving resolution of approximately 500 nm with minimal background signal across depths of at least 500 μm through a brain slice.

Furthermore, a significant advantage of UCNPs for AO technique in achieving high-resolution deep tissue imaging is their flexible multiple emissions through diverse ion-doped [147], thus offering a wide range of options for optimizing the imaging depth and resolution in various applications. That said, the primary challenge in direct-wavefront-sensing AO predominantly arises from the difficulty in capturing clear GS images deep within biological tissue. We anticipate that the imaging depth can be further extended by utilizing GS-used emissions at an even longer wavelength, which can be excited by upconversion effect or downconversion effect [148] with various ion doping, thereby enhancing the detectability of wavefront sensing.

Beyond the outstanding resolution in challenging depths, a standout feature of this technique is the low light toxicity provided by the CW laser excitation. This significantly diminishes photobleaching and photodamage due to the low-power CW laser excitation, enabling long-term dynamic tracking with a wavelength-scale resolution in deep tissue. Additionally, this technique can be synergistically integrated with other microscopy techniques as an add-on feature to enhance penetration depth while maintaining resolution, using only a low-cost CW laser excitation. This exceptional extensibility offers significant convenience for different tissue imaging requirements, such as finer resolution or deeper penetration depth.

Overall, HDU-AO provides a robust solution for high-resolution imaging in deep tissue. This technique holds great promise for a wide range of biomedical applications. It allows for detailed and long-term monitoring of biological structures with low-cost and low-toxicity CW excitation, enabling researchers to explore the intricacies of biological structures with unprecedented clarity and precision at most challenging depth.

4 SPECKLE-BASED UPCONVERSION NONLINEAR SUPER-RESOLUTION OPTICAL FLUCTUATION IMAGING

This chapter is reproduced with some adaptations according to a published journal paper: *Yao J, Yu Z, Gao Y, et al. Speckle-based upconversion nonlinear super-resolution optical fluctuation imaging. Submitted.*

4.1 Introduction

Recently, researchers have turned to inherent nonlinear optical responses of fluorescent probes to further enhance spatial resolution in super-resolution techniques, exemplified by nonlinear structured-illumination microscopy (NL-SIM) [81,149-153], two-photon stimulated emission depletion (TP-STED) [154,155], and NISM [156,157]. Since the excitation is confined to the focal plane, nonlinear super-resolution techniques not only provide intrinsically three-dimensional sectioning ability but also significantly mitigate background interference. However, conventional nonlinear fluorescent probes often suffer from intrinsic drawbacks such as strong photobleaching and photodamage, and typically require pulsed lasers to provide the high-intensity light necessary to induce nonlinear effects [158]. These limitations restrict their performance and reliability in demanding tissue imaging applications.

UCNPs have emerged as powerful tools in biomedical imaging due to their unique higher-order nonlinear optical response [145,159-163]. Under CW 975 nm NIR excitation, UCNPs absorb multiple low-energy NIR photons and emit higher-energy NIR or VIS photons [164,165]. The unique higher-order nonlinearity of UCNPs [133] underpins several fundamental advantages, such as the ability to utilize CW excitation to minimize photodamage and photobleaching, and the capacity to deliver sustained, non-blinking photostability from long-lived intermediate states [133,166-168]. Recent advances have leveraged these advantages to achieve significantly enhanced resolution and performance in a variety of nonlinear super-resolution techniques, including

nonlinear STED microscopy [93,127,169], NSIM [99], FED [95,96,135], and emission saturation microscopy [97,170], SMED microscopy [98] and super-linear microscopy [100].

Notably, the application of SOFI [54,62,171,172] based on UCNPs remains unexplored despite the many advantages UCNPs offer for nonlinear super-resolution imaging. SOFI enhances spatial resolution and image contrast by statistically analyzing temporal fluctuations in fluorescence signals, offering advantages such as operational simplicity, compatibility with standard imaging systems, superior noise rejection, and tunable resolution enhancement through the adjustment of statistical orders. However, a major challenge emerges when employing optical fluctuation methods for super-resolution imaging with nonlinear upconversion probes, as they do not exhibit the fluorescence blinking required by conventional SOFI.

To overcome these limitations, chapter presents a novel speckle-based upconversion nonlinear SOFI (SUN-SOFI) approach. In this approach, speckle patterns with random intensity distributions are generated via a spatial light modulator, which in turn induces dynamic fluctuations within the imaging system. These fluctuations effectively replicate the temporal intensity variations conventionally produced by fluorescence blinking in traditional SOFI. Leveraging these speckle-induced fluctuations, our approach provides a controlled and robust mechanism to achieve nonlinear SOFI without requiring intrinsic blinking from UCNPs. Experimental investigations on both nanoparticle and cellular samples demonstrate that SUN-SOFI can resolve multiple points beyond the diffraction limit while significantly enhancing the imaging SNR. In summary, our SUN-SOFI method offers a versatile super-resolution imaging platform that integrates the unique nonlinear properties of UCNPs with the enhanced resolution of SOFI, thereby providing a practical super-resolution tool for improved nanoscale biological imaging.

4.2 Methods and Materials

4.2.1 Optical Setup

The SUN-SOFI microscope starts with a CW laser output that is expanded by a beam expander (AC254-030-AB; AC254-100-AB) and sent to a phase-only spatial light modulator (SLM, PLUTO-BB II, Holoeye Photonics). Random phase masks displayed on the SLM convert the flat wavefront into a speckle field with well-defined statistical properties. Lens L_1 (AC254-150-AB) focuses the modulated beam onto mirror M; together with lens L_2 (AC254-150-AB) this forms a 4f relay that faithfully reproduces the SLM phase at the dichroic mirror. The dichroic mirror applies a pre-computed compensation pattern that cancels residual system aberrations. The corrected beam is then focused by the objective lens (OL, UplanSApo 60x NA=1.2, OLYMPUS) onto the sample, where upconversion nanoparticles generate visible fluorescence under near-infrared excitation. The emitted fluorescence is collected by the same OL, separated from the excitation path by a dichroic, and imaged by lens L_3 (AC508-500-AB) onto an sCMOS camera (Dhyana 400BSI, Tucsens). A stack of speckle-illuminated frames is processed with higher-order cumulant analysis to yield a SUN-SOFI super-resolution image.

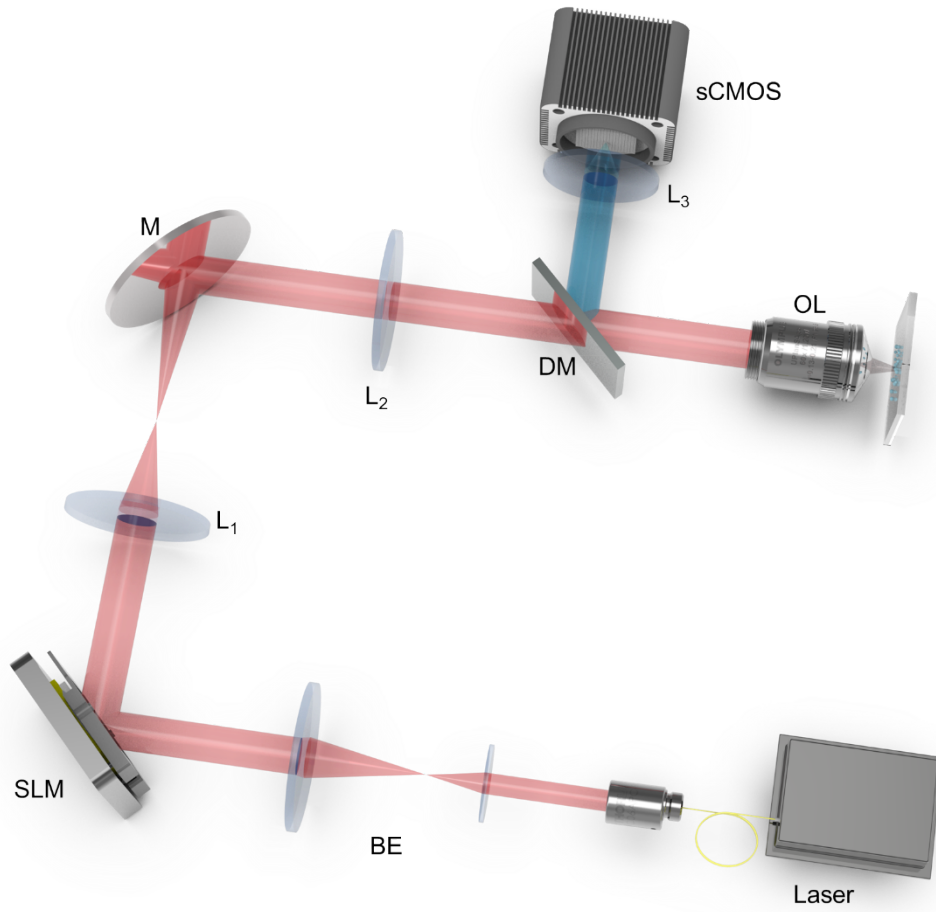


Fig. 4-1 Optical configuration for generating dynamic speckle patterns and capturing images of UCNPs. BE: beam expender; M: mirror; DM: dichroic mirror; OL: objective lens.

4.2.2 Principle of SOFI

Consider a sample containing N fluorescent emitters located at positions $\mathbf{r}_k$, the resulting fluorescence source distribution is given by

$$\sum_{k=1}^n \delta(\mathbf{r} - \mathbf{r}_k) \varepsilon_k s_k(t), \quad (4-1)$$

where ε_k is the constant molecular brightness and $s_k(t)$ is a time-dependent fluctuation.

If we denote the PSF of the imaging system as $\mathbf{U}(\mathbf{r})$, the fluorescence intensity $\mathbf{F}(\mathbf{r}, t)$ measured at a spatial position $\mathbf{r}_k$ and time t is given by:

$$\mathbf{F}(\mathbf{r}, t) = \sum_{k=1}^N U(\mathbf{r} - \mathbf{r}_k) \boldsymbol{\varepsilon}_k \mathbf{s}_k(t). \quad (4-2)$$

The time-averaged intensity at position $\mathbf{r}$ is calculated as:

$$\langle \mathbf{F}(\mathbf{r}) \rangle = \mathbf{1}/T \sum_{t=1}^T \mathbf{F}(\mathbf{r}, t), \quad (4-3)$$

where $\langle \rangle$ denotes time averaging. Intensity fluctuations are thus defined as deviations from this temporal average:

$$\delta \mathbf{F}(\mathbf{r}, t) = \mathbf{F}(\mathbf{r}, t) - \langle \mathbf{F}(\mathbf{r}) \rangle. \quad (4-4)$$

Substituting the expression for $\mathbf{F}(\mathbf{r}, t)$, the fluctuations become:

$$\delta \mathbf{F}(\mathbf{r}, t) = \sum_{k=1}^N U(\mathbf{r} - \mathbf{r}_k) \boldsymbol{\varepsilon}_k \delta \mathbf{s}_k(t), \quad (4-5)$$

where $\delta \mathbf{s}_k(t) = \mathbf{s}_k(t) - \langle \mathbf{s}_k(t) \rangle$, and has a temporal mean of zero.

The n th-order correlation function is given by:

$$\mathbf{G}_n(\mathbf{r}, \boldsymbol{\tau}_1, \dots, \boldsymbol{\tau}_{n-1}) = \langle \delta \mathbf{F}(\mathbf{r}, t) \delta \mathbf{F}(\mathbf{r}, t + \boldsymbol{\tau}_1) \cdots \delta \mathbf{F}(\mathbf{r}, t + \boldsymbol{\tau}_{n-1}) \rangle. \quad (4-6)$$

However, to obtain higher-order SOFI images, the n th-order correlation functions must be converted into their corresponding n th-order cumulant functions $\mathbf{C}_n(\mathbf{r}, \boldsymbol{\tau}_1, \dots, \boldsymbol{\tau}_{n-1})$, which isolate single-emitter contributions and eliminate cross-terms arising from multiple emitters. It can be written as

$$\mathbf{C}_n(\mathbf{r}, \boldsymbol{\tau}_1, \dots, \boldsymbol{\tau}_{n-1}) = \sum_{k=1}^N U^n(\mathbf{r} - \mathbf{r}_k) \boldsymbol{\varepsilon}_k^n \mathbf{w}_k(\boldsymbol{\tau}_1, \dots, \boldsymbol{\tau}_{n-1}), \quad (4-7)$$

where $\mathbf{w}_k(\boldsymbol{\tau}_1, \dots, \boldsymbol{\tau}_{n-1})$ denotes a correlation-based weighting function that reflects the specific temporal fluctuation characteristics of each emitter.

Assuming that the original PSF of the optical system can be approximated by a 3D Gaussian distribution, it follows from Eq. 4-6 that the effective PSF width in the n -th order SOFI image is reduced by a factor of $\sqrt{n}$ along all spatial dimensions, thereby enhancing the achievable optical resolution:

$$U(\mathbf{r}) = \exp\left(-\frac{x^2+y^2}{2\omega_0^2} - \frac{z^2}{2\omega_{z0}^2}\right), \quad (4-8)$$

$$U^n(\mathbf{r}) = \exp\left(-\frac{x^2+y^2}{2\tilde{\omega}_0^2} - \frac{z^2}{2\tilde{\omega}_{z0}^2}\right), \quad (4-9)$$

where $\tilde{\omega}_0 = \omega_0/\sqrt{n}$, $\tilde{\omega}_{z0} = \omega_{z0}/\sqrt{n}$.

4.2.3 Principle of SUN-SOFI

Conventional SOFI exploits the intrinsic temporal fluctuations of fluorophores, namely stochastic blinking, to achieve super-resolution. Under CW excitation, UCNPs can emit stable, long-lived fluorescence. While this stability improves *in vivo* imaging by enhancing photostability and reducing photodamage and photobleaching, it abolishes the temporal variability required for conventional SOFI method. To overcome this limitation, we substitute the intrinsic fluorophore blinking with externally induced fluorescence fluctuations generated by dynamic speckle illumination. In our optical setup (Fig. 4-1), the sample is sequentially illuminated by time-varying random speckle patterns produced by a spatial light modulator using random phase masks. These speckle patterns introduce random temporal variations in the local excitation intensity, thereby mimicking the stochastic on-off behavior of blinking fluorophores and satisfying the statistical prerequisites for SOFI reconstruction (Fig. 4-2).

Under dynamic speckle illumination, the fluorescence signal $\mathbf{F}(\mathbf{r}, t)$ at position $\mathbf{r}$ and time t is given by the convolution of the collective system's point-spread-function (PSF) $U(\mathbf{r})_{coll.}$ and the fluorescence source distribution

$$\mathbf{F}(\mathbf{r}, t) = \sum_{k=1}^N U(\mathbf{r} - \mathbf{r}_k)_{coll.} \varepsilon_k \mathbf{S}_k(t). \quad (4-10)$$

Here ε_k denotes the brightness of the k -th emitter and $\mathbf{S}_k(t)$ is the fluctuation. In SUN-SOFI, $\mathbf{S}_k(t)$ term which represents fluctuation that does not come from intrinsic fluctuation of fluorophores, but from induced fluctuation using speckle patterns illumination. Therefore, we must consider not only fluctuation of fluorophores induced by varying speckle patterns illumination, but also their correlation.

As an example, the second order cumulant function $\mathbf{G}_2(\mathbf{r}, \boldsymbol{\tau})$ in the case of a sample containing two fluorophores, based on equations (4-10), is given by

$$G_2 = \sum_{j,k}^N U(\mathbf{r} - \mathbf{r}_j)_{coll.} U(\mathbf{r} - \mathbf{r}_k)_{coll.} \epsilon_j \epsilon_k C_m(\mathbf{r}_k - \mathbf{r}_j), \quad (4-11)$$

where $C_m(\mathbf{r}_k - \mathbf{r}_j)$ represents the cumulant correlation functions, which can be written as [173]

$$C_m(\mathbf{r}_k - \mathbf{r}_j) = A \left| U(\mathbf{r}_k - \mathbf{r}_j)_{UC5}^2 \right|. \quad (4-12)$$

Here $U(\mathbf{r}_k - \mathbf{r}_j)_{UC5}$ is the effective excitation PSF in our method. In this nonlinear upconversion process, the excitation of upconversion fluorescence is a 5-photon process, thus the emission PSF can be expressed as

$$U(\mathbf{r})_{UC5} = U(\mathbf{r})_{illu.}^5, \quad (4-13)$$

where τ is the time lag between each frame. Based on Equation (4-13), Equation (4-12) can be rewritten as

$$C_m(\mathbf{r}_k - \mathbf{r}_j) = A \left| U(\mathbf{r}_k - \mathbf{r}_j)_{UC5}^2 \right| = A \left| U(\mathbf{r}_k - \mathbf{r}_j)_{illu.}^{10} \right|, \quad (4-14)$$

where A is the constant decided by the imaging system, then Equation (4-11) becomes

$$G_2 = \sum_{j,k}^N U(\mathbf{r} - \mathbf{r}_j)_{coll.} U(\mathbf{r} - \mathbf{r}_k)_{coll.} \epsilon_j \epsilon_k \cdot A \left| U(\mathbf{r}_k - \mathbf{r}_j)_{illu.}^{10} \right|. \quad (4-15)$$

In Equation (4-15), because the emission PSF depends on the fifth power of the illumination PSF in our method, higher-order cumulants generate even higher powers of $U_{illu.}$. This mechanism further enhances resolution compared with linear fluorescence processes.

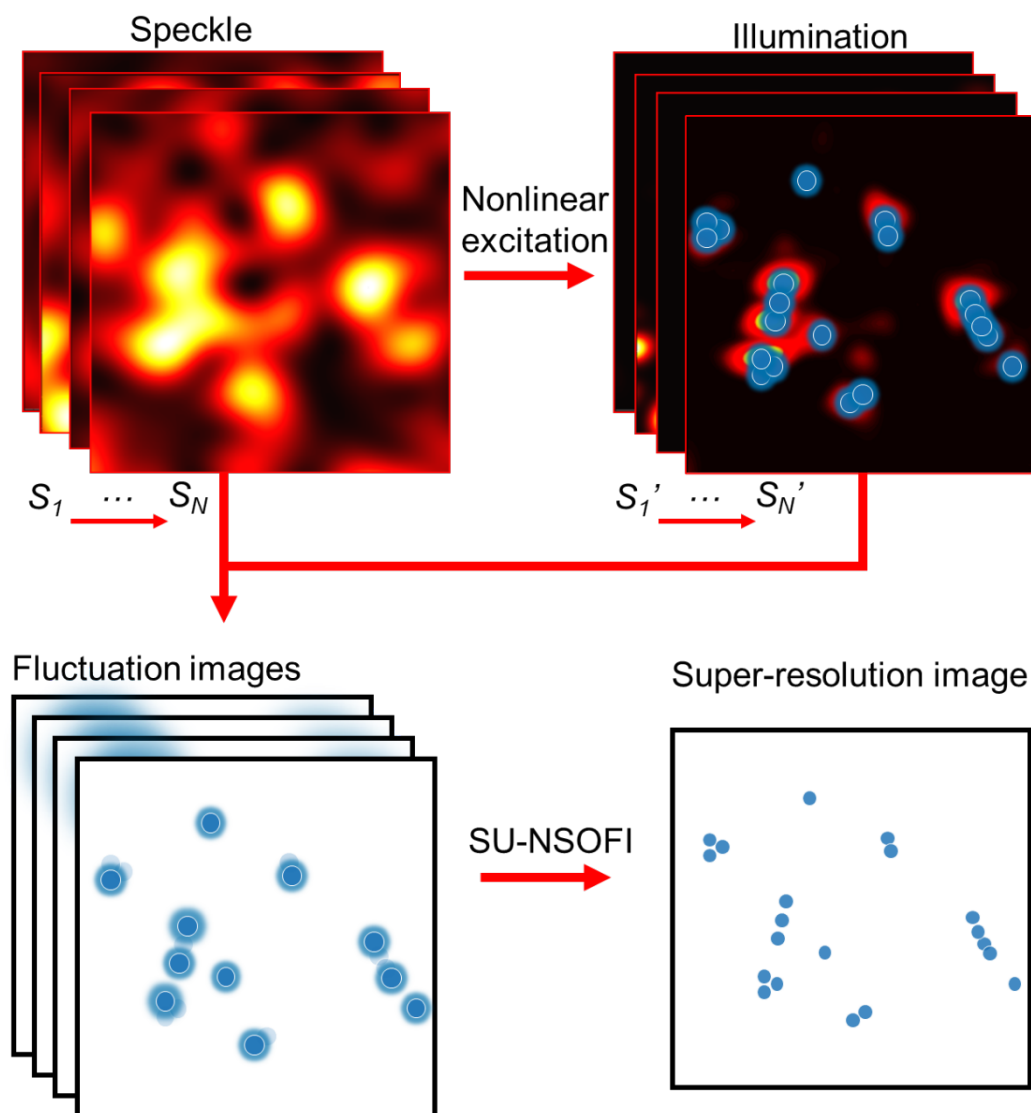


Fig. 4-2 Schematic diagram illustrates the principle of SUN-SOFI.

To assess morphology and size uniformity of the synthesized Tm^{3+} Yb^{3+} co doped UCNP, transmission electron microscopy (TEM) was performed (Fig. 4-3a). Analysis of all particles in this TEM image yields an average diameter of 33 nm with a standard deviation of about 4 nm, confirming a narrow size distribution essential for consistent optical performance and super-resolution imaging.

Under continuous wave 975 nm excitation the emission spectrum is dominated by an intense peak at 455 nm arising from the $^1\text{D}_2 \rightarrow ^3\text{F}_4$ transition of Tm^{3+} (Fig. 4-3b). The inset energy-level diagram illustrates that energy absorbed by Yb^{3+} sensitizers sequentially transfer up to the $^1\text{D}_2$ state of Tm^{3+} before radiative decay produces the

455 nm emission. The UCNPs used in this chapter were synthesized following the same protocol as described in Chapter 3, except that the Tm^{3+} doping concentration was varied as the only differing parameter. By varying the Tm^{3+} concentration from 0.5 mol% to 8 mol%, we observe that emission intensity increases as more activator centers become available. The optimum brightness is achieved at 8 mol% Tm^{3+} , a balance that maximizes output under moderate pump power densities and ensures high SNR for nonlinear imaging.

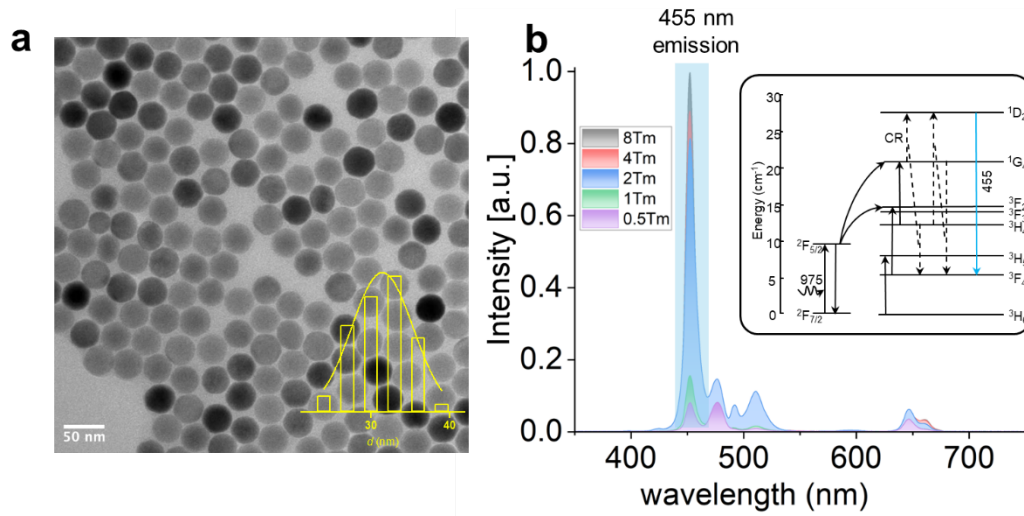


Fig. 4-3 Characteristics of the utilized UCNPs co-doped with Tm^{3+} and Yb^{3+} . (a) TEM images of utilized UCNPs, whose average size is ~ 33 nm. Scalebar, 50 nm. (b) Upconversion emission spectrum of UCNPs with different Tm^{3+} concentration under 975 nm excitation and the energy diagram and upconversion process of Yb^{3+} and Tm^{3+} co-doped UCNPs.

The nonlinear response of the UCNPs with 8 mol% Tm^{3+} was quantified by measuring the 455 nm emission intensity over a pump power density range from approximately 10^3 to $10^4 \text{ kW} \cdot \text{cm}^{-2}$. In a log–log plot of emission versus pump intensity (Fig. 4-4), the data fit a straight line with a maximum slope of 4.77. This indicates that nearly 5-photons participate in the upconversion process. Such a high multiphoton order causes the emission probability to scale very steeply with local excitation intensity, yielding two principal effects. First, the effective excitation PSF narrows substantially compared with a linear emitter because of fifth-order nonlinearity, which in turn reduces speckle grain size and confines emission to the highest-intensity regions. Second, out-of-focus

and low-intensity speckle fluctuations are effectively suppressed, improving the signal-to-noise ratio and the efficiency of cumulant calculation. Together, these effects enable finer spatial modulation in speckle-based imaging and directly translate into enhanced spatial resolution.

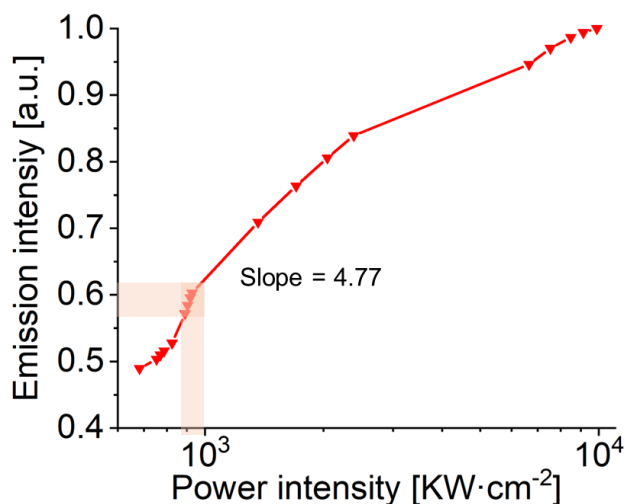


Fig. 4-4 The excitation intensity-dependent emission curve (nonlinearity). Slope = 4.77.

4.3.4 Photostability of UCNPs

To substantiate the suitability of UCNPs for SOFI, we first verified their photostability under continuous NLSM illumination for 60 min (excitation power density 0.5 MW cm^{-2} at 980 nm), as shown in Fig. 4-5. Twelve consecutive TIFF frames were recorded, each comprising $128 \times 20\,000$ pixels and covering exactly 300 s (each pixel 150 μs). This configuration yields an effective sampling interval of 100 μs per pixel, where the additional 50 μs beyond the 100 μs dwell time accounts for line fly-back and synchronization overhead. After concatenating the frames into a $12 \times 128 \times 20,000$ matrix and averaging along the spatial dimension, we obtained a per-pixel emission-intensity time trace. Panel a display the full 0–60 min trajectory, where most points cluster tightly around 3000 ± 350 counts and sporadic outliers stem from shot-noise fluctuations. A magnified view of the initial 10–15 min segment, highlighted by the brown rectangle, is shown in Fig. 5b. Statistical comparison between this segment and the entire data set reveals no significant differences in mean intensity, standard

deviation, or skewness. These findings indicate the absence of detectable photobleaching under the applied CW illumination, confirming that UCNPs possess superior photostability relative to conventional organic dyes and are well suited for prolonged in-vivo visualization.

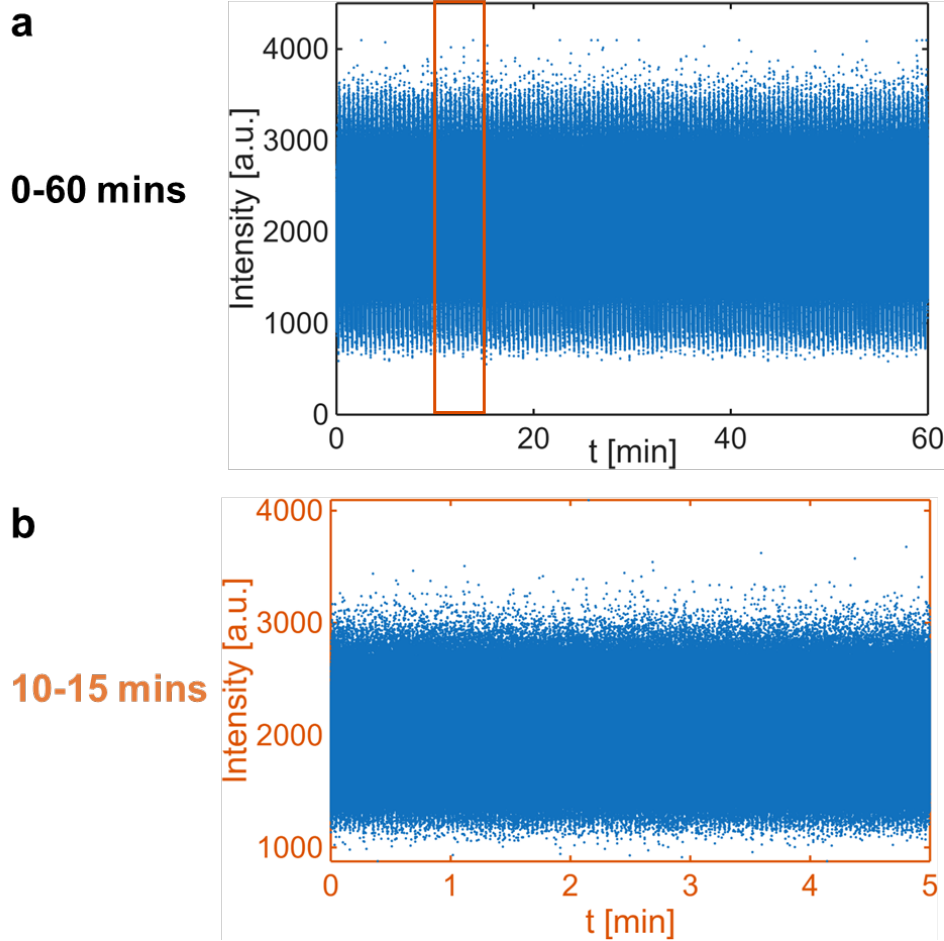


Fig. 4-5 Photostability assessment of UCNPs under continuous NLSM illumination. (a) Scatter plot of single-pixel emission intensities recorded over 60 min (sampling interval 100 μ s). The brown rectangle marks the 10–15 min. **(b)** Enlarged view of the 10–15 min segment reveals intensity statistics comparable to the full data set, confirming the absence of significant photobleaching.

4.3 Results and Discussion

4.3.1 Nonlinear Speckle Simulation

To investigate the nonlinear speckle characteristics, we conducted numerical

simulations based on predefined system parameters. Initially, the linear speckle pattern was generated at the sample plane, as illustrated in Fig. 4-6a. This speckle distribution exhibits a typical random intensity pattern formed due to the coherent interference of multiple scattered optical fields. We subsequently simulated the 5th-order nonlinear excitation process. The resulting speckle pattern after this nonlinear interaction is depicted in Fig. 4-6b.

Compared to the linear speckle pattern, the nonlinear speckle features significantly reduced intensity and sharper localization. This outcome aligns with the theoretical prediction that higher-order nonlinear excitation emphasizes the high-intensity regions and dramatically suppresses background contributions, thus facilitating enhanced resolution and contrast in imaging applications. This simulated nonlinear speckle pattern corresponds to the scheme presented in Fig. 4-2b, effectively reducing the illumination area on the sample. Such selective 5th nonlinear random illumination significantly improves spatial resolution and signal-to-noise ratio, making it particularly advantageous for high-resolution imaging.

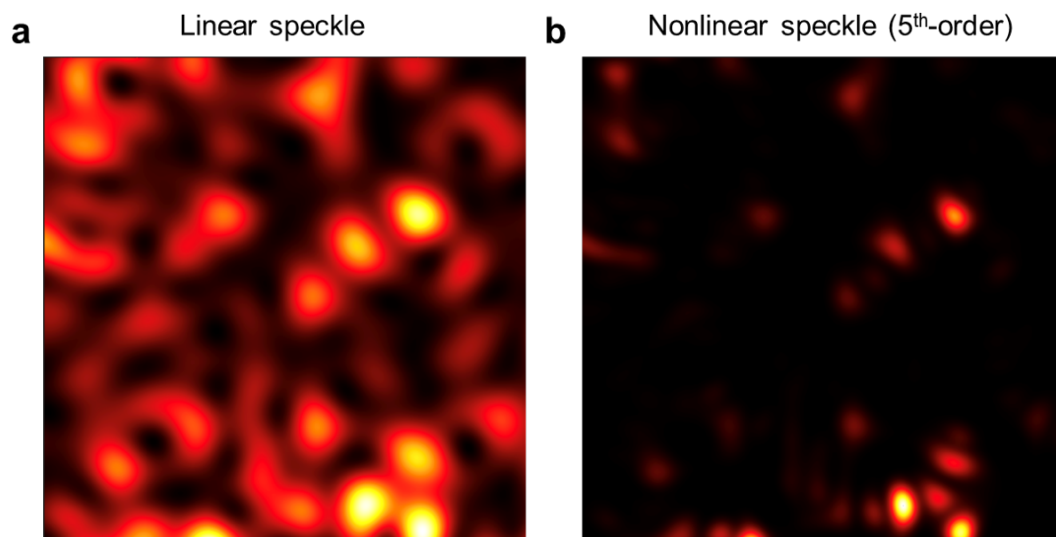


Fig. 4-6 Simulated Speckles. (a) Simulated linear speckle pattern exhibiting a typical random intensity distribution generated through numerical modeling. (b) Speckle pattern after 5th-order nonlinear excitation.

4.3.2 SUN-SOFI Reconstruction of UCNPs Sample

To evaluate the performance of the SUN-SOFI method, we imaged a UCNPs sample sparsely distributed on a microscope coverslip. Figure 4-7 presents a comparative analysis of conventional wide-field imaging (Mean), deconvolution, and SUN-SOFI reconstructions at various cumulant orders, with particular emphasis on the 6th-order reconstruction. For a fair comparison, the average of the speckle pattern illuminations used for reconstruction serves as an appropriate surrogate for uniform illumination. The wide-field (Mean) image exhibits both poor SNR and limited resolution. Although deconvolution significantly enhances the SNR, the diffraction limit still hinders the separation of closely spaced, overlapping nanoparticles. In contrast, the 6th-order SUN-SOFI reconstruction not only markedly improves the SNR but also substantially enhances the resolution, enabling the discrimination of nanoparticles that were previously unresolved according to the Rayleigh criterion.

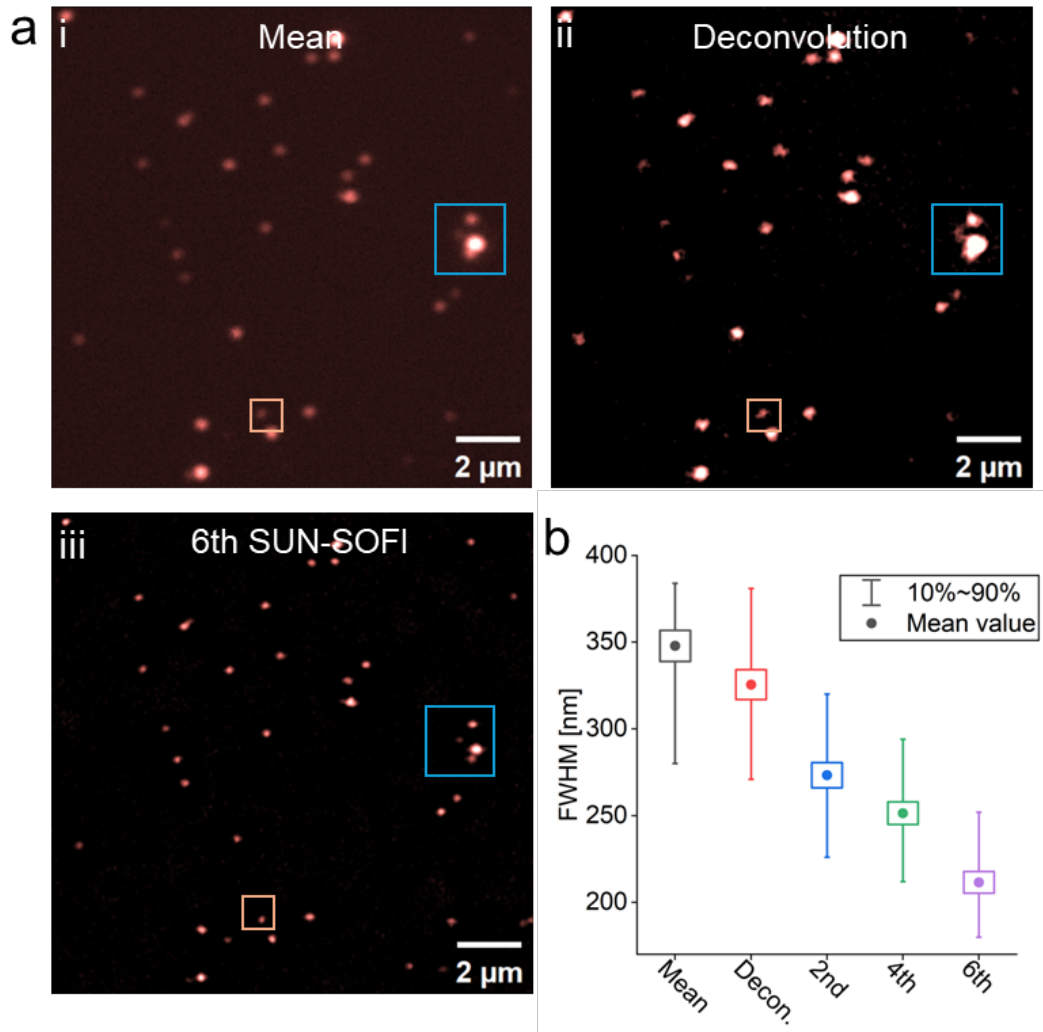


Fig. 4-7 Comparison of UCNPs images achieved by SUN-SOFI relative to conventional wide-field (Mean) imaging and deconvolution. (a) Representative UCNPs images under (i) wide-field illumination (Mean), (ii) deconvolution, and (iii) 6th-order SUN-SOFI; the orange and blue boxes indicate regions chosen for detailed analysis. **(b)** Statistical distributions of full-width at half-maximum (FWHM) values for images reconstructed via (i) Mean, (ii) deconvolution, (iii) 2nd-order, (iv) 4th-order, and (v) 6th-order SUN-SOFI.

To further illustrate the resolution enhancement, we focus on a pair of UCNPs separated by a distance below the diffraction limit (Fig. 4-8). Here, images processed by deconvolution as well as by 2nd-, 4th-, and 6th-order SUN-SOFI cumulants are shown. With increasing cumulant order, the effective PSF progressively narrows. The corresponding cross-sectional intensity profiles (indicated by the green dotted line) demonstrate that while the deconvolved and lower-order reconstructions fail to resolve

the two nanoparticles, the 6th-order SUN-SOFI image distinctly separates them, thereby effectively overcoming the diffraction limit.

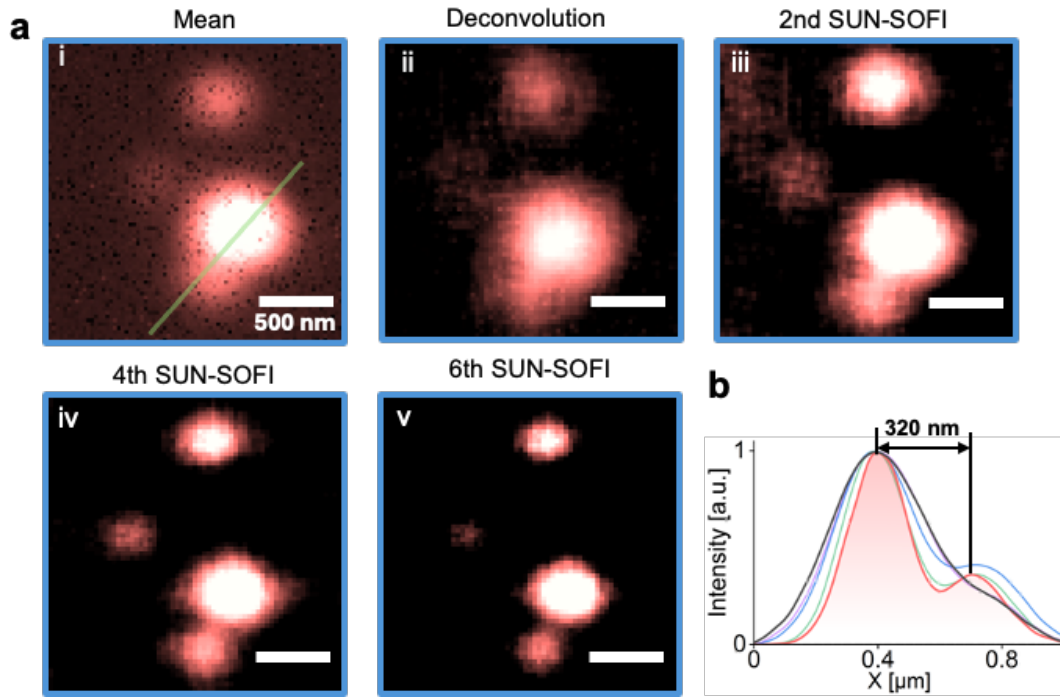


Fig. 4-8 Enlarged view of the orange-boxed region showing images obtained by mean, deconvolution, as well as 2nd-order, 4th-order, and 6th-order SUN-SOFI.

Quantitative analysis of resolution is provided in Fig. 4-9. Here, the PSF of the raw wide-field (Mean) image was fitted with a one-dimensional Gaussian function, yielding a full width at half maximum (FWHM) of 280 ± 2 nm. In contrast, the 6th-order SUN-SOFI reconstruction reduces the FWHM to approximately 160 nm. These results collectively indicate that SUN-SOFI represents a significant advancement in super-resolution imaging, offering promising potential for applications requiring precise nanoscale resolution.

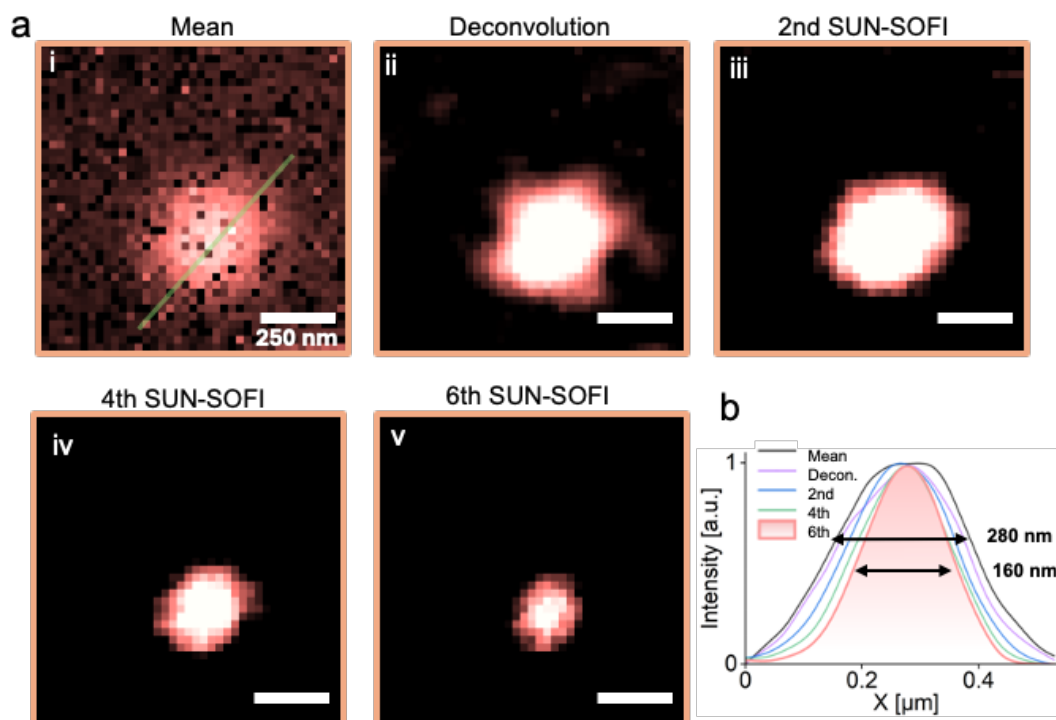


Fig. 4-9 Enlarged view of the blue-boxed region showing images obtained by Mean, deconvolution, 2nd-order, 4th-order, and 6th-order SUN-SOFI.

4.3.3 SUN-SOFI Reconstruction of Hela Cells

To demonstrate the applicability of the SUN-SOFI method in biological imaging, Hela cells labeled with UCNPs were imaged. Figure 4-10 displays the wide-field (Mean) illumination image (i), along with the SUN-SOFI reconstructions at 2nd (ii), 3rd (iii), and 6th (iv) orders. Compared to the wide-field image, the SUN-SOFI results exhibit significant improvements in spatial resolution and SNR, primarily due to the suppression of uncorrelated background signals. Notably, in the high-order (6th) SUN-SOFI reconstruction, several closely spaced nanoparticles, which appear as blurred, merged clusters in the wide-field (mean) image, are clearly resolved as distinct points.

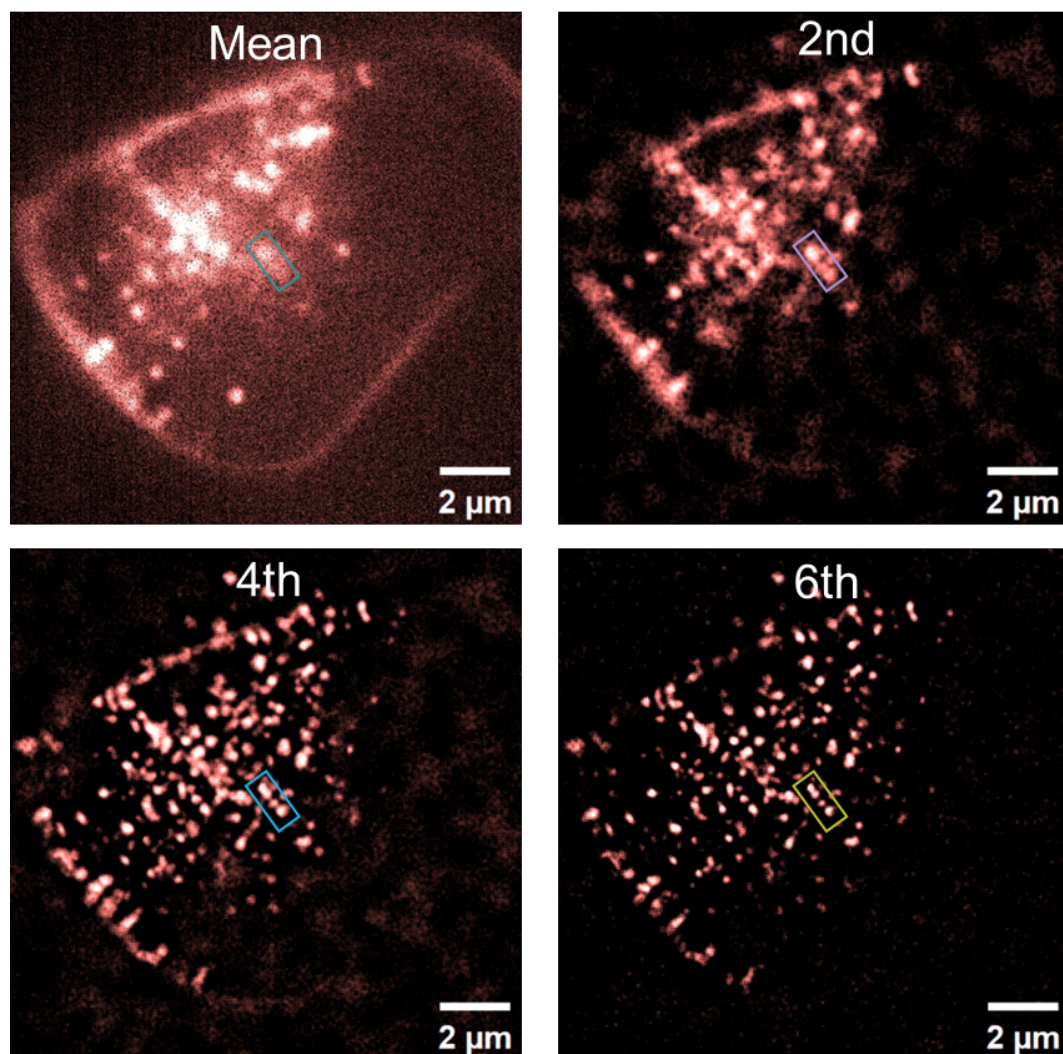


Fig. 4-10 Comparison of cell images labeled with UCNPs with different orders of SUN-SOFI reconstruction relative to conventional wide-field (Mean) imaging.

Figure 4-11 provides a detailed comparison of a selected region of interest. In the magnified view, four closely positioned nanoparticles that are barely discernible in the wide-field (mean) image are clearly separated in the 6th-order SUN-SOFI reconstruction, with the corresponding intensity profiles indicating a significant enhancement in contrast and resolution. Additionally, the intensity profiles along the lines in Fig. 4-11 indicate that while the raw image exhibits blurred and merged features, the SUN-SOFI reconstruction reveals well-separated and sharper nanoparticles. Notably, the minimum resolvable separation between two nanoparticles is approximately 270 nm. These results demonstrate that SUN-SOFI can effectively

enhance the visualization of subcellular structures in UCNP-labeled samples without relying on intrinsic fluorescence blinking.

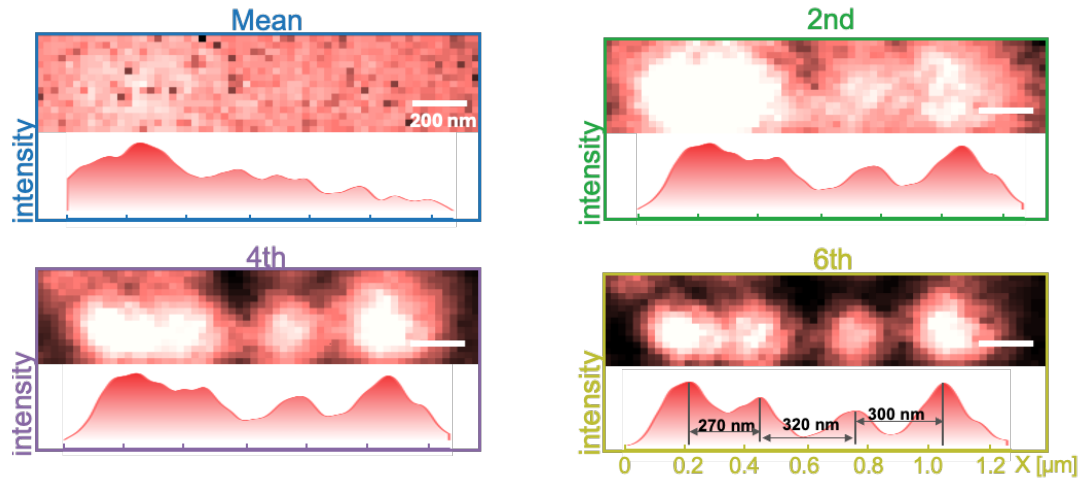


Fig. 4-11 Magnified view of the ROI with corresponding cross-sectional intensity profiles, demonstrating the enhanced resolution and improved SNR achieved by SUN-SOFI compared to conventional wide-field imaging.

4.4 Summary

This chapter introduced SUN-SOFI, a speckle-based upconversion SOFI technique for UCNPs that leverages controllable temporal fluctuations to achieve super-resolution. A SLM projects diffraction-limited speckle patterns that drive nonlinear upconversion excitation, resulting in a twofold improvement in spatial resolution over conventional wide-field imaging. Sixth-order cumulant reconstruction yields an effective resolution of approximately 160 nm. In cell experiments, SUN-SOFI reduces background in tissue-like samples and further sharpens resolution, enabling dynamic tracking of intracellular events at the single-nanoparticle scale [174].

This innovation extends the applicability of SOFI to upconversion imaging, effectively overcoming the inherent limitation of UCNPs that lack intrinsic fluorescence blinking. Under CW excitation, UCNPs exhibit an approximately fifth-order nonlinear response. This high-order nonlinearity counteracts resolution loss introduced by the speckle PSF and its correlations, and using UCNPs with even stronger nonlinear excitation can

further boost SUN-SOFI's resolving power. Importantly, SUN-SOFI requires only a SLM to generate disordered speckle patterns, making it trivially compatible with existing upconversion microscope setups.

While SUN-SOFI delivers marked improvements in spatial resolution, there remains scope for further refinement. The need to acquire a large number of frames extends both acquisition time and computational load; this requirement can be eased by incorporating two-step convolution algorithms [62]. Moreover, although fifth-order nonlinear excitation of UCNPs sharpens illumination PSF, the ultimate resolution is still constrained by the diffraction-limited size of the speckle patterns determined by the objective lens. To overcome this bound, one may employ UCNPs with higher-order nonlinear responses or use objectives with larger NAs to generate finer speckle grains.

Despite these improvements, SUN-SOFI still shares certain fundamental limitations with conventional SOFI when extended to higher-order cumulant analysis. While the resolution enhancement theoretically scales as $\sqrt{n}$ scaling with cumulant order n , the practical application of higher-order SOFI is constrained by several factors. First, the signal-to-noise ratio (SNR) declines rapidly with increasing order due to the amplification of both signal fluctuations and background noise. Second, higher-order cumulants require substantially more frames for statistical convergence, which is often impractical in live-cell imaging scenarios. Third, fluorescence bleaching and non-stationary blinking behaviors can introduce bias into cumulant estimation, leading to artifacts and degraded structural accuracy. Additionally, the computational complexity of reconstructing higher-order SOFI images increases significantly, demanding greater processing time and memory. Finally, although high-frequency spatial details are amplified, overall image contrast often decreases, making structural interpretation more challenging. These collective limitations restrict the practical use of SOFI beyond the fourth or fifth order. In this study, sixth-order reconstruction was adopted as a compromise between resolution enhancement and image fidelity.

Looking ahead, we aim to further extend the SUN-SOFI framework toward even higher-order implementations. By incorporating advanced statistical denoising

strategies and adaptive photon budget control, we hope to overcome the aforementioned limitations and unlock deeper resolution gains. Ultimately, SUN-SOFI stands out as a promising and accessible super-resolution technique for UCNP-based microscopy. Its minimal hardware requirements and low phototoxicity make it particularly attractive for live-cell imaging applications. Moreover, the use of near-infrared excitation offers unique advantages in deep-tissue microscopy, enabling greater penetration depth and high-fidelity subsurface imaging. Future work will focus on adapting SUN-SOFI for real-time, single-particle super-resolution imaging in vivo, thereby enabling dynamic investigations of molecular and cellular processes deep within living tissues.

5 DISCUSSION AND FUTURE WORK

5.1 Major Findings

This thesis is aimed to advance the frontiers of upconversion nanoparticle (UCNP)-enabled nonlinear microscopy by integrating novel optical engineering, computational imaging, and nanomaterial innovations. Collectively, these contributions overcome persistent limitations of conventional nonlinear optical microscopy (NLOM)—including high excitation thresholds, depth-resolution trade-offs, and phototoxicity—enabling high-resolution deep-tissue and super-resolution imaging under continuous-wave (CW) near-infrared excitation.

Key innovations include:

Real-time adaptive optics (AO) for aberration correction (Chapter 2): We developed an AO nonlinear microscope that exploits UCNPs as in situ GS and contrast agents. In this system, embedded UCNPs emit narrowband fluorescence under NIR CW excitation that serves both as a bright imaging signal and as a direct GS. This approach overcomes the limitations of conventional fluorescent probes (e.g. photobleaching and pulse laser requirement). By measuring and correcting sample-induced aberrations on the UCNPs fluorescence, the AO system successfully refocuses distorted light, yielding tightly focused spots with large intensity enhancement with serious aberrations.

Homologous dual-emission microscopy (HDU-AOM) (Chapter 3): We introduced a homologous dual-emission UCNPs strategy combined with AO to simultaneously maximize penetration depth and resolution under CW excitation. Specifically, $\text{Tm}^{3+}/\text{Yb}^{3+}$ co-doped UCNPs were designed to emit at 800 nm (NIR) and 455 nm (VIS) under 975 nm CW excitation. The 800 nm (NIR) emission served as a deep-tissue GS for aberration sensing, while the 455 nm emission provided high-resolution fluorescence imaging at the same challenging depth. Using a custom NLSM and AO correction based on the UCNPs GS, we achieved near-diffraction-limited imaging (lateral resolution ~ 480 nm) at 500 μm depth in mouse brain environment. This

performance (large penetration depth with high resolution and SNR under CW excitation) is unprecedented for nonlinear fluorescence, highlighting the effectiveness of dual-emission UCNPs in breaking the depth-resolution compromise.

Speckle-based super-resolution (SUN-SOFI) (Chapter 4): We developed a robust speckle-based SOFI method using non-blinking UCNP labels. Since UCNPs do not exhibit intrinsic on/off blinking, we induced controlled fluorescence fluctuations by illuminating the sample with dynamic nonlinear random speckle patterns. This artificially modulated excitation generated intensity fluctuations that, when analyzed via higher-order cumulants, yielded sub-diffraction-limited resolution. Crucially, the UCNPs' outstanding photostability and lack of photobleaching ensured consistent signal and high throughput throughout the acquisition. The resulting "SUN-SOFI" images showed clear resolution enhancement over diffraction-limited widefield images without relying on blinking dyes or complex switching schemes.

Taken together, these results demonstrate the unique advantages of UCNPs as multifunctional probes for deep-tissue and higher/super-resolution imaging. We have shown that UCNPs' stable, narrowband emissions can serve dually as wavefront GS and imaging, enabling AO corrections that penetrate deeper and resolve finer details than conventional methods. The homologous dual-emission approach pushes the envelope of CW-excited nonlinear microscopy, while the speckle-induced fluctuation strategy leverages non-blinking UCNPs' stability for robust super-resolution imaging. Collectively, this work establishes that UCNP-based modalities can significantly extend the capabilities of optical bioimaging, paving the way for high-resolution studies in complex biological environments. Engineering the emission spectrum, such as introducing new dopants or energy transfer schemes, could yield multiple wavelengths or switching capabilities for more versatile imaging. Surface chemistry and biocompatible coating should be refined to improve colloidal stability and reduce toxicity for *in vivo* use.

5.2 Limitation Discussion

Despite the improvements introduced in this thesis, several limitations remain that hinder broader application. These limitations arise from two main aspects: the intrinsic properties of UCNPs and the challenges of biological targeting.

Accumulated evidence indicates substantial intrinsic limitations of UCNPs in terms of optical efficiency, fluorescence lifetime, and biocompatibility. First, the external quantum yield of individual UCNPs typically remains below 1%, rendering them significantly dimmer than conventional nonlinear fluorophores. This low brightness imposes limitations on detection sensitivity, particularly in deep-tissue imaging or rapid volumetric scanning. Second, the upconversion emission lifetimes of Tm^{3+} -doped UCNPs are in the range of hundreds of microseconds, which is several orders of magnitude longer than most cellular dynamics. Such long-lived emission leads to motion blur in live-cell imaging and imposes constraints on achievable frame rates, thereby limiting the accurate observation of fast physiological events. Third, to enhance brightness and reduce surface quenching, especially due to surface hydroxyl ($-\text{OH}$) groups, UCNPs are often synthesized as core-shell structures with heavy doping. However, these designs typically increase particle size beyond 20 nm, which not only compromises renal clearance but also risks long-term accumulation in the reticuloendothelial system. Upon shell degradation, the release of free lanthanide ions may also raise concerns regarding chronic toxicity. These intrinsic drawbacks in brightness, temporal resolution, and biosafety currently limit the utility of UCNPs for real-time high-resolution deep imaging.

Beyond the intrinsic optical constraints of UCNPs, biological targeting presents additional obstacles that stem largely from three main factors. First, their relatively large hydrodynamic size (often > 20 nm even after surface engineering) reduces membrane permeability, which in turn lowers labeling density at sub-cellular targets and can trigger size-dependent cytotoxic responses. Second, the overall targeting efficiency of UCNPs remains low, meaning that only a small fraction of the injected

particles reaches the intended biological site. Third, although many coupling chemistries exist, the palette of high-affinity ligands that remain stable and functional on UCNP surfaces is still limited; as a result, achieving dense, oriented grafting of antibodies, peptides, or aptamers without compromising colloidal stability is non-trivial.

5.3 Future Work

Building on these advances, future investigations will focus on overcoming the current limitations of UCNPs while deepening the AO, SOFI, and other imaging techniques, thereby expanding the scope and impact for UCNP-based nonlinear microscopy. Potential opportunities include:

UCNP design improvements: Future work should concentrate on four interconnected goals: enhancing fluorescence brightness, lowering the excitation threshold, shortening the fluorescence lifetime and enhancing biological compatibility. Brightness can be enhanced by optimizing sensitizer-activator ratios and adopting protective core-shell layouts [175], surface coordination of molecules [176]. A reduced threshold will come from engineering the local optical environment, such as adding thin photonic coatings [177]. Upconversion lifetime can be shortened by tailoring energy-transfer pathways [178], fabricating a tilted plasmonic nanocavity [179]. Shrinking overall hydrodynamic size and grafting simple targeting ligands to improve clearance and cell specificity.

Biological targeting of UCNPs: To overcome current limitations in the biological targeting of UCNPs, future research is focusing on several complementary strategies. First, optimizing UCNP particle size and shape can significantly enhance cellular uptake, as smaller nanocarriers and those with rod-like (high aspect ratio) morphologies tend to penetrate cell membranes more efficiently [180]. Second, the design and conjugation of high-affinity targeting ligands (such as antibodies, peptides, or aptamers) onto the nanoparticle surface is pursued to confer selective binding to target cells, thereby improving the specificity and accumulation of UCNPs at diseased sites [181]. Third, advanced surface functionalization techniques, including PEGylation, zwitterionic coatings, or biomimetic cell-membrane cloaking, are employed to improve

colloidal stability and reduce nonspecific protein adsorption, effectively masking the particles from immune recognition [182]. Finally, to achieve efficient intracellular delivery, researchers are incorporating membrane-penetrating peptides and endosomal escape mechanisms that allow UCNP (and their payloads) to breach cellular barriers and avoid endo-lysosomal entrapment, thus increasing the fraction of nanoparticles that successfully release cargo into the cytosol [183].

AO and SOFI imaging algorithms: AO algorithms and SOFI imaging can be enhanced for speed and robustness. For AO algorithms, this includes implementing digital AO technique [184] and machine-learning methods [185] to rapidly optimize the wavefront in scattering samples. Multi-conjugate AO [186] or extended depth-of-field techniques could further expand the corrected volume. For SOFI imaging, advanced computational imaging algorithms can be integrated to extract more information from the UCNP samples and to reduce residual noise.

Multimodal integration: Except for nonlinear fluorescence luminescence, upconversion nanoparticles can be reformulated into truly multimodal agents that combine MRI and photoacoustic (PA) read-outs within the same nanoplatfrom[187,188]. For MRI, paramagnetic ions such as Gd^{3+} or Mn^{2+} can be incorporated into the crystal lattice [188], or superparamagnetic iron-oxide domains can be co-encapsulated [189]. For PA imaging, attaching NIR absorbers such as indocyanine-green analogues or Ag_2Se nanocrystals [190] to the particle surface converts the same probe into an efficient thermoelastic emitter, enabling centimeter-deep visualization by ultrasound while the upconversion channel simultaneously reports molecular information. By combining these complementary contrasts, a single UCNP probe can deliver functional and molecular data within one acquisition, greatly enriching *in vivo* imaging studies.

In summary, future development will concentrate on refining UCNP probes, AO, and SOFI methods while developing novel imaging techniques and exploring biological applications. By advancing the brightness, specificity of UCNP, and the design of UCNP-based nonlinear microscopy techniques, it will be possible to achieve deeper,

faster, and more precise biological imaging. Such developments will help translate UCNP-based nonlinear microscopy from proof-of-concept demonstrations toward practical tools for biomedical research and potentially clinical diagnostics.

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