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Phase angle encoded upconversion luminescent nanocrystals for multiplexed applications

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Lanthanide-doped upconversion nanoparticles (UCNPs) are increasingly used as luminescent candidates in multiplexed applications due to their excellent optical properties. In the past, several encoding identities have been proposed for UCNPs, including emission colour, intensity ratio between different emission bands, colour spatial distribution, and luminescence lifetime. In this paper, a new optical encoding dimension for upconversion nanomaterials is developed by exploring their luminescence kinetics, i.e., the phase angle of upconversion luminescence in response to a harmonic-wave excitation. Our theoretical derivation shows that the phase angle is governed jointly by the rise and decay times, characterizing the upconversion luminescence kinetics. Experimentally, a full set of methods are developed to manage the upconversion luminescence kinetics, through which the rise and decay times can be manipulated dependently or independently. Furthermore, a large phase-angle space is achieved in which tens of unique codes can be potentially generated in the same colour channel. Our work greatly extends the multiplexing capacity of UCNPs, and offers new opportunities for their applications in a wide range such as microarray assays, bioimaging, anti-counterfeiting, deep tissue multiplexed labelling/detection and high-density data storage. In addition, the development of this luminescence kinetics-based optical encoding strategy is also instructive for developing multiplexing techniques using other cascade luminescent systems that inherently lack multi-spectral channels, such as triplet-triplet annihilation molecule pairs.

1. Introduction

Optical multiplexing has applications in broad areas including information technologies^{1,2}, high-throughput clinical diagnostics³⁻⁵, multichannel bioimaging⁶ and anti-counterfeiting⁷, as it enables high data-storage capacity, simultaneous identification and quantification of multiple species, multiple target registration, improved encryption, and so on. In optical multiplexing techniques, the optical encoding dimensions play a fundamental role, in which multiple unique codes are generated. During the past decades, many optical encoding dimensions have been developed, including wavelength⁴, polarization¹, spatial dimensions⁸ lifetime⁹⁻¹³ and angular momentum², largely extending the general multiplexing capacity of optical techniques. Even so, it has always been of high interest to develop new encoding dimensions to meet the

increasing requirements of various applications. For instance, for implementing stimulated emission depletion (STED) microscopy (where a normal Gaussian excitation beam and a doughnut shaped emission depletion beam are used coaxially to achieve high spatial resolution¹⁴) in multi-channels, non-spectral multiplexing strategies are desired due to the challenge of aligning multiple beams¹⁵, and this demand is not yet met.

Optical multiplexing is generally performed with luminescent materials, such as fluorescent dyes and semiconductor quantum dots. As most frequently used fluorophores, organic dyes suffer from drawbacks such as photobleaching, spectral overlap, and the need for multiple light sources to excite different species. Quantum dots offer significant advantages over organic fluorophores because of their excellent chemical-/photo-stability and excitability by a single wavelength, typically in the blue or ultraviolet region. However, applications of quantum dots are hampered by their toxicity as well as the short excitation-wavelength leading to fairly strong background fluorescence from substrates. Thus, the development of appropriate luminescent materials for multiplexed applications has attracted strong interest.

An attractive alternative group of luminescent materials for optical multiplexing is upconversion nanoparticles (UCNPs), generally comprised of an inorganic host doped with lanthanide ions¹⁶. UCNPs are capable of converting low-intensity near-infrared (NIR) excitation light to narrow-band visible emission¹⁷, and have excellent chemical-/photo-stability and nil-autofluorescence noise¹⁸⁻²³. Due to these properties, UCNPs are increasingly used in

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*Electronic Supplementary Information (ESI) available: theoretical analysis on the impulse response function of upconversion dynamic system and the connection of the decay and rise time constants to the phase angle, numerical simulations of the phase angle, nanoparticle syntheses, fabrication of polymeric beads and films, inkjet printing of UCNPs, luminescence color decoding, portable phase angle measurement system, principle of separation of multiple groups of phase-encoded UCNPs by performing frequency-domain measurements. See DOI: 10.1039/x0xx00000x

multiplexing applications. Inherited from the studies on fluorescent dyes and quantum dots, optical multiplexing strategies using UCNPs have been developed in the spectral domain^{7, 24-27}, time domain²⁸ and spatial domain^{29, 30}. However, studies of the complex luminescence mechanisms involving sequential multi-step photophysical and energy transfer processes featuring complex luminescence kinetics are still at their infancy³¹⁻³⁴, and may open possibility to develop unique multiplexing strategies in other domains.

In this work, we report a new multiplexing concept using UCNPs by manipulating their luminescence kinetics to create an optical encoding dimension in the frequency domain, i.e., the phase angle of upconversion luminescence in response to harmonic-wave excitation. Theoretically, we prove that this phase angle feature is determined by both the noticeable rise and decay times, characterizing the upconversion luminescence kinetics following short pulse excitation. Experimentally, we demonstrate that the rise and decay times can be engineered dependently or nearly independently by making use of doping concentration effect, energy migration effect, quenching ion effect and surface quenching effect, and thus that the phase angle can be efficiently tuned in a large range, allowing for a large number of unique codes. The decoding method is user-friendly, as the phase angle can be accurately retrieved using a cost-effective and miniaturized frequency-domain lifetime measurement system. This phase-angle coding enriches the library of optical encoding dimensions, and particularly offers new opportunities for multiplexing applications of UCNPs in a wide range, as demonstrated by anti-counterfeiting and deep tissue multiplexed labelling/detection in this work. In addition, the developed luminescence kinetics-based optical encoding technique may potentially benefit applications where non-spectral "multicolor" strategies are preferred, e.g., in STED microscopy.

2. Experimental

2.1 Nanoparticle synthesis

NaYF₄-based UCNPs were synthesized following previously reported protocols with modifications^{35, 36}. Further experimental details are provided in the Electronic Supplementary Information (ESI).

2.2 Nanoparticle characterization and photoluminescence measurements

Transmission electron microscopy (TEM) images were recorded on a Jeol 2010F transmission electron microscope (Jeol Ltd.) operating at an acceleration voltage of 200 kV. Upconversion spectra were recorded on an Acton SpectroPro 2300i spectrophotometer (Princeton Instruments) equipped with an NIR 2 W continuous-wave laser at 980 nm (EINST Technology Pte. Ltd.). In the upconversion luminescence kinetics measurements, a digital-pulse generator (TGP110, TTI) was used to modulate the 980 nm laser to generate the short pulse excitation, and the signal was detected by a photomultiplier tube (Hamamatsu R928) coupled to a digital storage oscilloscope (Tektronix TDS2024C).

2.3 Phase angle characterization

Phase angle measurements of upconversion nanocrystal suspensions were carried out on a frequency-domain fluorescence

lifetime measurement system based on an Acton SpectroPro 2300i spectrophotometer (Princeton Instruments). An NIR 2 W continuous-wave laser at 980 nm (EINST Technology Pte. Ltd.), sinusoidally modulated at a specific modulation frequency by a SFG-2120 synthesized function generator (GW INSTEK), was used to provide excitation light. The scattered excitation light profile was monitored by a photodetector (DET10A, Thorlabs), and the emission light profile was monitored by a photomultiplier tube (Hamamatsu R928) equipped with the spectrometer. Both the excitation and emission profiles were recorded on a digital storage oscilloscope (Tektronix TDS2024C). After correction on the system delay, including time delays in air, in the cables and electronic circuits, the two profiles were fitted with cosine functions using least-squares algorithm, with the modulation frequency as the fixed parameter and others (offset, amplitude and phase) as free parameters. The phase angle of the emission relative to the excitation was extracted by taking the subtraction of the returned phases. Phase angle measurements in double encryption studies and *in vivo* deep tissue labelling study were carried with a bifurcated fiber-probe based portable system (Supplementary Schematic S3 and Supplementary Section 5 in the ESI).

2.4 *In vivo* deep tissue labeling

This study conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, USA and protocol approved by the Institutional Animal Care and Use Committee (IACUC), National University of Singapore. The experiments used a 5 weeks old nude mouse and anesthesia was done by intraperitoneal injection of ketamine (75 mg/kg body weight) and medetomidine (1 mg/kg body weight). *In vivo* imaging following intramuscular administration of 0.1 mg of three different groups of NIR emitting (800 nm) UCNPs was performed using the μ Fluor-980 small animal upconversion luminescence imaging system (Einst Technology Pte Ltd., Singapore). Briefly, 0.1 mg of silica-coated UCNPs in 50 μ L saline was injected intramuscularly at three different sites. Imaging was performed 10 min after administration of the UCNPs, under 980 nm laser excitation delivering a fluence rate equivalent to 250 mW/cm². The phase angle of the UCNPs was measured using the portable phase delay measurement setup as described in Supplementary Section 5 in the ESI.

3. Results and discussion

3.1. The luminescence kinetics of UCNPs following short pulse excitation and their phase angle in response to a harmonic-wave excitation

The most efficient upconversion luminescence is achieved by sequential energy transfer upconversion (ETU) process, involving two types of ions, acting as sensitizers and activators, respectively¹⁸. In the upconversion process, the sensitizers successively transfer absorbed light energy to a neighboring activator ion, pumping the activator ion to its upper emitting state, where anti-Stokes shifted emission is generated, as illustrated in Fig. 1a by a model two-photon upconversion process. Due to the nature of sequential discrete absorption of two or more excitation photons, the upconversion luminescence kinetics following a short pulse

excitation typically features a delayed maximum in the time trace rather than immediate decay, reflecting both rise and decay behavior (Fig. 1b)^{37,38}. Such luminescence kinetics can generally fit adequately to the following equation³⁹⁻⁴¹:

$$I_{\delta}(t) = A \left[\exp\left(-\frac{t}{\tau_d}\right) - \exp\left(-\frac{t}{\tau_r}\right) \right], \quad (1)$$

where A is a scaling constant, and $\tau_{d(r)}$ is the decay (rise) time constant. It should be clarified that the upconversion luminescence as the target output of UCNP, its rise and decay times are the temporal features of the whole upconversion system, rather than intrinsic radiative properties of any individual energy state. Our theoretical analysis based on a simplified rate equation model reveals that both the rise and decay times are impacted by the lifetimes of the involved energy states and also by the energy transfer effect between sensitizers and activators (Supplementary Section 1 in the ESI). Under weak-excitation assumptions, the decay time constant largely reflects of the lifetime of the activator's upper emitting state, while the rise time constant is mainly governed by

the lifetimes of the sensitizer's excited state and the activator's intermediate state (Supplementary Section 1 in the ESI).

Under the harmonic-wave excitation, the upconversion luminescence is forced to respond with the same frequency but with a phase lag, and the modulation depth will be varied (Fig. 1c)⁴². It can be derived that the phase angle (φ_u) for upconversion luminescence is given by (Supplementary Section 2 in the ESI):

$$\varphi_u = \arccos \left[\frac{1 - \omega^2 \tau_d \tau_r}{\sqrt{(1 - \omega^2 \tau_d \tau_r)^2 + \omega^2 (\tau_d + \tau_r)^2}} \right], \quad (2)$$

where ω is the modulation circular frequency. As indicated, the phase angle of upconversion luminescence is governed by both the rise and decay times. Calculated results show that upconversion luminescence has phase angle easily exceeding 90° and approaching 180° (Fig. 1d), in stark contrast to downconversion fluorophores, the phase angle of which is some fraction of 90° independent of the modulation frequency (Fig. 1d), given by $\varphi_d = \arccos(1/\sqrt{1 + \omega^2 \tau_d^2})$ ⁴². Such difference indicates the importance of the rise time in governing the phase angle of the upconversion luminescence.

The phase angle of upconversion luminescence was also studied by numerical simulations to mimic the harmonic-wave response of a practical upconversion system involving complex energy transfer processes, e.g., the $\text{Yb}^{3+}/\text{Er}^{3+}$ codoping system (Supplementary Section 3 in the ESI). The simulated results confirm the easy accessibility of $>90^\circ$ phase angle of upconversion luminescence, exemplified by the two-photon green upconversion luminescence (at 543 nm), while that of downconversion luminescence (at $\sim 1 \mu\text{m}$) remains below 90° under the same condition (Fig. S1 in the ESI). The significantly enlarged phase angle space of the UCNP is also confirmed by subsequent experimental findings.

Based on these findings, we propose the phase angle of upconversion luminescence as an encoding dimension of individual UCNP.

3.2 Management of upconversion luminescence kinetics for tunable phase angles

In order to generate plenty of unique codes to satisfy the need of multiplexing applications, a broad range of phase angles is necessitated. As indicated by equation (2), this can be accomplished by manipulating the rise and decay times of the upconversion luminescence. Next, we aimed to develop a full set of kinetics-management methods for upconversion luminescence to yield tunable phase angles. Recent advances in the synthetic methods have allowed easy access to UCNP with well-defined core-(multi)shell structures. We started with using core-multishell structures in order to acquire large flexibility for tuning of the upconversion process. Based on the functions of different layers in the nanostructure, they are distinguished as pumping layer, emitting layer, and quenching layer, respectively, as illustrated in Figure 2a. The emitting layer as the kernel part is the conventional sensitizer-activator-homogeneously-codoped region, where upconversion luminescence is eventually generated. The pumping layer (containing only sensitizer ions) and quenching layer (containing only quenching ions) are optional, and if incorporated, they act to absorb excitation light energy and migrate to the sensitizer ions located in the emitting layer and to quench the upper emitting state of the activator ions, respectively. Our idea is

to tune the rise and decay behaviors of upconversion luminescence through controlling the energy transfer among doped optical centers within the emitting layer or across different functional layers.

3.2.1. Tuning the emitting layer

We initiated the luminescence kinetics-management study by manipulating the homogeneously-doped emitting layer by means of the doping concentration effect, given that upconversion luminescence is highly dependent on dopant concentrations^{28,43}. A typical core-shell structure, $\text{NaYF}_4\text{:x}\%\text{Yb}^{3+},\text{y}\%\text{Er}^{3+}\text{@NaLuF}_4$, was

Figure 2. Tuning the emitting layer, pumping layer and quenching layer to generate phase-angle encoded UCNPs. **a**, Schematic design of the nanostructure for kinetics-management of upconversion luminescence. **b**, The influence of the dopant concentrations in the emitting layer on the temporal behaviors of 543 nm upconversion luminescence (Upper) and the resulting phase angles (Lower). **c**, The influence of the Yb³⁺ concentration in the pumping core on the temporal behaviors of 543 nm upconversion luminescence (Upper) and the resulting phase angles (Lower). **d**, The influence of the Yb³⁺ concentration in the isolated pumping core on the temporal behaviors of 543 nm upconversion luminescence (Upper) and the resulting phase angles. **e**, The influence of the Nd³⁺ concentration in the quenching layer on the temporal behaviors of 543 nm upconversion luminescence (Upper) and the resulting phase angles (Lower). **f**, The large phase-angle space achieved through kinetics-management of upconversion luminescence.

employed in this study (Fig. S2 in the ESI). We note that an optical inert layer (NaLuF_4) was added as the outermost part in order to isolate the surface quenching effect. This isolating strategy was adopted through this work for all UCNP s except when surface quenching effect was intentionally employed. The effect of Yb^{3+} concentration was first investigated. UCNP s with fixed Er^{3+} concentration (2%) and varied Yb^{3+} concentration (20% or 60%) were synthesized using a previously reported protocol^{35,44}, and the temporal behavior of their green upconversion luminescence (at 543 nm) following short pulse excitation (20 μs pulse width, 100 Hz repetition rate) was studied. As shown in Fig. 2b, with higher Yb^{3+} concentration, the green upconversion luminescence exhibits both faster rise and decay. Results of studies on UCNP s with high Er^{3+} concentration (8%) also show that a higher Yb^{3+} concentration induces a faster temporal behavior (Fig. 2b). This Yb^{3+} concentration effect can be attributed to two factors: (a) higher Yb^{3+} concentration generally leads to a decreased lifetime of the Yb^{3+} excited state⁴⁵, resulting in a shorter rise time of upconversion luminescence; (b) higher Yb^{3+} concentration causes more efficient $^4\text{S}_{3/2}(\text{Er}^{3+}) \rightarrow ^2\text{F}_{7/2}(\text{Yb}^{3+})$ energy back transfer^{46,47}, resulting in faster decay of the green emission. The faster temporal response to excitation light caused by the higher Yb^{3+} concentration led to a smaller phase angle in response to harmonic-wave excitation at all modulation frequencies tested (10 – 4000 Hz), as shown in Fig. 2b.

Studies on UCNP s with varied Er^{3+} concentration (2% or 8%) and fixed Yb^{3+} concentration (20%) reveal that an elevated Er^{3+} concentration could exert more complicated influence on the temporal behavior of the green upconversion luminescence. As shown in Fig. 2b, with increasing Er^{3+} concentration from 2% to 8%, the green luminescence exhibits faster rise but slower decay behavior. As to the resulting phase angles, interestingly, the UCNP s with higher Er^{3+} concentration (8%) exhibit larger phase angle than those with lower Er^{3+} concentration (2%) at modulation frequencies less than 200 Hz, but exhibit smaller phase angle at modulation frequencies above 300 Hz (Fig. 2b). The phase-angle results are well correlated with the time traces. Note that the rise time of upconversion luminescence (typically tens of microseconds) is generally significantly shorter than the decay time (typically hundreds of microseconds)³⁷. At low modulation frequency, the period of the excitation harmonic wave is relatively long, and thus the comparatively longer decay time plays a dominant role over the rise time in determining the phase angle. Thus, the UCNP s with higher Er^{3+} concentration which has longer decay time possess larger phase angle than the UCNP s with lower Er^{3+} concentration. In contrast, at high modulation frequency equivalent to a short excitation wave period, the comparatively shorter rise time plays a dominant role in determining the phase angle over the decay time. Hence, the UCNP s with lower Er^{3+} concentration which has longer rise time exhibit a larger phase angle than the ones with higher Er^{3+} concentration. These results clearly show that both the rise and decay times play important roles in determining the phase angle and both could be the limiting factor at specific excitation conditions. Results of studies of UCNP s with high Yb^{3+} concentration (60%) and varied Er^{3+} concentration (2% or 8%) also show that a higher Er^{3+} concentration induced slower decay process (Fig. 2b). The slowed decay property might be explained by the decreased effective number of Yb^{3+} ions surrounding each Er^{3+} ion, which is

beneficial for suppressing the $^4\text{S}_{3/2}(\text{Er}^{3+}) \rightarrow ^2\text{F}_{7/2}(\text{Yb}^{3+})$ energy back transfer. Different from studies on UCNP s containing 20% Yb^{3+} ions, here higher Er^{3+} concentration induced a slower rise process (Fig. 2b).

Next, we also investigated the effect of codoping quenching ions, Nd^{3+} ions, given that Nd^{3+} ions can efficiently quench the Er^{3+} excited state⁴⁸⁻⁵¹. The results show that even a relatively low doping level (3%) can cause more rapid rise and decay properties (Fig. 2b), leading to significantly declined phase angles (Fig. 2b).

Taken together, concentration tuning in the emitting layer is an efficient strategy to tune the temporal behavior of upconversion luminescence, and is able to tune the phase angle in over a large range. Particularly, fast response UCNP s (with short rise and decay times), possessing small phase angles in response to a harmonic-wave excitation, can be readily achieved by high doping.

3.2.2 Tuning the pumping layer

Upconversion process is generally delocalized, involving fast and efficient energy migration among sensitizer ions⁵². Such long-range energy transfer effect opens new possibility for managing the time attributes of upconversion luminescence and thus tuning the phase angle by incorporating and manipulating extra pumping layer(s) (containing solely Yb^{3+} ions) in the nanostructures (Fig. 2c), which was explored in this study. A series of UCNP s with one additional pumping core, $\text{NaYF}_4:z\%\text{Yb}^{3+}@\text{NaYF}_4:10\%\text{Yb}^{3+},2\%\text{Er}^{3+}@\text{NaLuF}_4$ ($z = 0, 20, 40, 70$), were first synthesized (Fig. S3 in the ESI), and their temporal responses to short pulse excitation (20 μs , 100 Hz) were studied. The results show that the incorporation of the pumping core slowed the rise process of the upconversion luminescence, as shown in the inset of Fig. 2c. This may be ascribed to the larger potential energy-hopping region (i.e., the Yb^{3+} network) after adding the pumping core, leading to longer energy-hopping distance. The delayed rise process led to larger phase angle under harmonic-wave excitation at modulation frequencies higher than 1200 Hz (Fig. 2c). As to the decay process, $\text{NaYF}_4:0\%\text{Yb}^{3+}@\text{NaYF}_4:10\%\text{Yb}^{3+},2\%\text{Er}^{3+}@\text{NaLuF}_4$ UCNP s exhibit multi-exponential decay behavior, while others display nearly mono-exponential decays (Fig. 2c). It is worth noting that when increasing the Yb^{3+} concentration in the pumping core from 20% to 100% both the rise and decay processes become faster (Fig. 2c), leading to decreased phase angle at all modulation frequencies tested (Fig. 2c). This can be attributed to the difference in the radiative properties of Yb^{3+} in the pumping core caused by concentration difference, given that higher Yb^{3+} concentration results in shorter excited state lifetime⁴⁵. Such difference was apparently able to propagate to the emitting layer and influence the temporal behaviors of the eventual upconversion luminescence.

In order to gain more insights about the effect of the long-range energy migration, we extended the extra pumping region to two layers.

$\text{NaYF}_4:m\%\text{Yb}^{3+}@\text{NaYF}_4:2\%\text{Yb}^{3+}@\text{NaYF}_4:2\%\text{Yb}^{3+},2\%\text{Er}^{3+}@\text{NaLuF}_4$ ($m = 0, 15, 40, 70$) UCNP s were accordingly synthesized (Fig. S4 in the ESI), noting only the Yb^{3+} concentration in the very core was varied (Fig. 2d). Here, we employed low Yb^{3+} concentration (2%) in outer layers, intended to get relatively large phase angle. Amazingly, the extra pumping core though isolated from the emitting layer by a 5-nm thick $\text{NaYF}_4:2\%\text{Yb}^{3+}$ layer (Fig. S5 in the ESI), was still able to

exert influence on the upconversion process. As shown in Fig. 2d, this extra pumping core retarded the rise process of upconversion luminescence, leading to larger phase angle at modulation frequencies higher than 2000 Hz (Fig. 2d), similar to the results of above studies on UCNPs with one-layered pumping core. Interestingly, the decay process was found to be marginally influenced (Fig. 2d), significantly different from UCNPs with one-layered pumping region (Fig. 2c). This difference can be ascribed to the effect of the isolating/bridging layer ($\text{NaYF}_4\text{:}2\%\text{Yb}^{3+}$), which preserved the microenvironment of Er^{3+} ions in the emitting layer by avoiding direct contact with Yb^{3+} ions in the very core. In addition, higher Yb^{3+} concentration in the pumping core again tended to cause faster upconversion rise process (Fig. 2d). These results reveal that manipulating the pumping layer is a feasible strategy to tune the temporal behaviors of upconversion luminescence and generate tunable phase angle. Particularly, the rise time can be nearly independently tuned by employing

multilayer structures, with the decay time untouched to large extent.

3.2.3 Tuning the quenching layer

Next, we included a quenching layer to the multilayer structure by incorporating Nd^{3+} ions in the outermost NaLuF_4 layer, and investigated how it could impact the temporal behaviors of upconversion luminescence. $\text{NaYF}_4\text{:}70\%\text{Yb}^{3+}\text{@NaYF}_4\text{:}2\%\text{Yb}^{3+}\text{@NaYF}_4\text{:}2\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}\text{@NaLuF}_4\text{:}n\%\text{Nd}$ ($n = 0, 30, 50$) UCNPs were synthesized (Fig. S6 in the ESI), and the time traces of the green upconversion luminescence at 543 nm following short pulse excitation were studied. As shown in Fig. 2e, with addition of Nd^{3+} ions, the rise process was just slightly influenced. However, Nd^{3+} ions caused apparently faster decay process, and such effect showed a positive correlation with the doping concentration (Fig. 2e). The induced faster decay process led to smaller phase angle (Fig. 2e). These results indicate that the

decay time of upconversion luminescence can be nearly independently tuned by manipulating the quenching layer.

Above on these explorations, we are able to create a large phase angle space spanning from $\sim 11^\circ$ to $\sim 111^\circ$ at the modulation frequency of 1000 Hz (Fig. 2f). With high accuracy in the phase-angle measurement with a standard deviation typically less than 2° , such a space can accommodate tens of unique codes in the green emitting channel.

3.2.4. Utilizing surface quenching effect

The approaches outlined above employing multi-layer structures are demanding in synthesis, and those involving the concentration effect are inevitably at the price of luminescence intensity due to deviations from optimized levels of the doping concentration. In view of this, we developed a simple method to generate phase-angle encoded UCNP while maintaining high luminescence intensity, i.e., nanoparticle size adjustment regulated by optically-inert-ion doping. This method takes advantages of the inherent surface quenching effect for UCNP, i.e., that the lifetimes of involved states of both the sensitizers and activators are susceptible to environments^{43, 53, 54}. By employing the Lu^{3+} -doping regulating method⁵⁵, four groups of $\text{Yb}^{3+}/\text{Er}^{3+}$ codoped NaYF_4 nanocrystals (denoted as samples G1-G4) were synthesized, with fixed concentrations of 20% Yb^{3+} and 2% Er^{3+} . These samples have significantly different average nanocrystal diameters, ranging from 25 nm to 120 nm (Fig. S7 in the ESI). The time traces of the 543 nm upconversion luminescence following short pulse excitation at 980 nm (20 μs , 100 Hz) of these nanocrystals are presented in Fig. 3a. As expected, smaller sized nanocrystals exhibit shorter rise and decay times than larger ones due to the surface quenching effect⁴³. Fig. 3b presents the phase angles of these UCNP in response to the harmonic-wave excitation at various modulation frequencies. As seen, these UCNP display remarkably distinct phase angles in a large modulation frequency range (200 – 3000 Hz).

3.3 Susceptibility of phase angle to variations in experimental conditions

In order to assess if the phase angle is a reliable encoding dimension, its susceptibility to experimental-condition variations, e.g., change in modulation amplitude and offset, and nanoparticle concentration, was investigated. A moderate modulation frequency of 500 Hz was employed, and samples G1-G4 were used in this study. Importantly, it was found that the coefficients of variation (CVs) of the phase angle remain below 1.5% for each of these groups, even though the modulation amplitude varies across more than an order of magnitude (Fig. 3c). In response to the variation in the modulation offset, larger nanoparticles (G3 and G4) exhibited systematically decreasing phase angles with increasing modulation offsets (with fixed modulation amplitude) (Fig. 3d), with slightly larger CV (less than 5%). However, with precise control of the modulation offset, these UCNP populations can always be well distinguished. In addition, the phase angle was found to be independent on nanoparticle concentration (Fig. S8 in the ESI). This is important for real-life applications, otherwise minute changes in nanoparticle concentration could cause undesired shifts in the

phase angle, leading to erroneous results. Taken together, we find that the phase angle is a reliable encoding dimension.

Apart from the green colour UCNP, UCNP with other emission colours with distinct phase angles can be also readily obtained, e.g., $\text{Yb}^{3+}/\text{Tm}^{3+}$ -codoped NaYF_4 nanocrystals in the blue colour channel, as demonstrated in Fig. S9 and Fig. S10 in the ESI. Since the colour and phase-angle encoding dimensions are orthogonal, the phase angle encoded UCNP will greatly complement the very limited number of efficient upconversion colour bands attainable, and increase the multiplexing capacity of UCNP-based techniques.

3.4 Colour and phase-angle hybrid optical encoding

The phase-angle discernible UCNP can be used solely as identity codes for optical encoding, and can be also used in combination of emission colours, which will enable the development of more advanced techniques, e.g., double encryption strategy. Here, we demonstrate a double encryption concept using UCNP-incorporated beads made of a photo-polymerizable polymer [polyethylene glycol diacrylate (PEG-DA)]. The beads encapsulating UCNP of different colours (green and blue) and phase angles were arranged in a rectangular array with certain patterns encrypted, as shown in Fig. 4a. In the decoding process, when the array was illuminated with a continuous-wave NIR laser, it showed a diagonal pattern of alternating green and blue beads (Fig. 4b). However, when the phase angle information was analyzed, deeperly encrypted information – “NUS” and “BME” (National University of Singapore, BioMedical Engineering Department), encoded with two specific phase angles, were extracted (Fig. 4c). It should be noted here that the green-colour beads were given a selectable offset in the phase angle (45° in this case) to match with the blue beads, because the two-photon green upconversion luminescence has significantly smaller phase angle than the three-photon blue luminescence (Fig. 3b and Fig. S10 in the ESI). This second-layer encryption (phase-angle encoding) associated with the freely assignable phase-angle offset will greatly increase the encoding/decoding complexity.

This double encryption strategy can be potentially used to make logos of luxury brands for protection from counterfeiting, where complex patterns other than bead arrays may be required. The encryption principle is generalized in Fig. 4d, where a pattern is designed to be composed of several different regions, and each of them is assigned to have certain emission colour (λ) and phase angle (ϕ). This strategy is demonstrated by an example using polymer films encapsulated with UCNP. A mould was made according to the design in Fig. 4d, and PDMS mixed with specific UCNP were loaded into different regions and polymerized to form a polymer film. When the film was decoded using a continuous-wave NIR laser, it showed a pattern of sun (green luminescence) in the sky (blue luminescence), as shown in Fig. 4e. However, when the phase angle information was analyzed, a sharp-contrast pattern of a new moon (phase angle ϕ_1 , green luminescence) in the sky was visualized (Fig. 4f). It is worthwhile mentioning that, apart from using the phase angle discernible groups of UCNP achieved previously, the beads could also be encapsulated with an UCNP mixture from two different groups to generate a phase angle feature in between (Fig. S11 in the ESI), which increases the flexibility of creating different phase angles.

Figure 4. Colour and phase-angle hybrid optical encoding for security purpose. **a**, Bright field image of PEG-DA beads encapsulated with different colour emitting and phase-angle upconversion nanocrystals, forming a rectangle array. **b**, This rectangle array is divided into three parts by examining the emission colour, two triangle regions with green colour and one rhomboid-like region with blue colour. **c**, The characters “NUS” and “BME” were encoded in the same array with two specific phase angles. Scale bar 10 mm. **d**, Design of the encoded pattern. The pattern is composed of three regions, characterized by the colour and phase-angle encoding indices (λ_1, φ_1) , (λ_1, φ_x) and (λ_2, φ_x) , respectively. λ_1 and λ_2 denote green colour and blue colour, respectively. φ_1 denotes the desired specific phase angle, while φ_x for other phase angle. **e**, A pattern of sun (green emission) is displayed in the sky (blue emission), when the pattern is decoded with a 980 nm continuous-wave laser. **f**, A completely opposite pattern of new moon (green emission with phase angle φ_1) in the sky (green or blue emission with other phase angles φ_x) is visible, when the phase angle information is analyzed. Scale bar 10 mm. **g**, The statue of liberty was printed using different colour UCNPs. Scale bar 5 mm. **h**, The gateway arch was printed with green emitting UCNPs of different phase angles in different regions, mapped with different pseudo colours. The inset shows the true-colour image. Scale bar 5 mm. **i**, The printed logo for US Federal reserve system was encoded using both colour and phase angle. The words were printed with green emitting UCNPs, and others were printed with blue emitting UCNPs. The second-layer information “IN GOD WE TRUST” was encoded with blue UCNPs having a specific phase angle mapped with pseudo violet colour. Scale bar 5 mm. The images in this figure are composite overlays from separate true-coloured and/or pseudo-coloured images.

The developed colour and phase angle hybrid encoding technology can be extended to other applications for security purpose, e.g., security printing. After removal of surface ligands these UCNPs can be readily printed using a commercially available inkjet printer, and thus used for encoding watermarks into documents and currencies. As a demonstration, we encoded watermarks in a US currency note using colour encoding, phase-

angle encoding and a combination of both in three different regions. The statue of liberty was encoded using luminescence colour in region ① (Fig. 4g), and the gateway arch was encoded using phase angle in region ② (Fig. 4h). The logo of the US Federal reserve system was encoded in region ③ using both emission colour and phase angle (Fig. 4i), where the blue and green represent true colours, and the violet is a phase-angle mapping

Figure 5. *In vivo* deep tissue labeling with phase encoded NIR emitting upconversion nanocrystals. **a**, Upconversion emission image at 800 nm of a nude mouse. The mouse was injected intra-muscularly three groups of NIR emitting (800 nm) silica-coated UCNPs, NaYF₄:30%Yb,2%Tm (20 nm), NaYF₄:30%Yb,2%Tm,30%Lu (34 nm) and NaYF₄:25%Yb,0.5%Tm (23 nm), respectively, at three different sites. **b**, The emissions from different spots can be efficiently resolved by their phase angles, although they have the same emission wavelength. Modulation frequency 500 Hz. Scale bar 10 mm.

pseudo colour. Such sophisticated methods of encoding watermarks into currency will add extra layers of security and help in preventing counterfeiting.

3.5 *In vivo* deep tissue multiplexed labelling

The phase angle encoding technique opens a new possibility for multiplexed labelling/detection in deep tissue *in vivo*, which could not be achieved previously because of the very few upconversion colour channels suitable for deep tissue studies (actually only the 800 nm emission band of Tm³⁺)⁵⁶. As a proof of concept, we have demonstrated that phase-angle encoded NIR-emitting (at 800 nm) UCNPs can be detected in deep tissue and resolved efficiently in a mouse. A nude mouse was intra-muscularly injected (at depths of ~3 mm) with three different groups of NIR-emitting UCNPs (NaYF₄:30%Yb, 2%Tm (20 nm), NaYF₄:30%Yb,2%Tm, 30%Lu (34 nm) and NaYF₄:25%Yb, 0.5%Tm (23 nm)) at three different sites. These UCNPs were coated with a dense SiO₂ layer before injection to gain biocompatibility. Subsequent luminescence imaging showed NIR luminescence at the same wavelength (800 nm) at the injection sites, as shown in Fig. 5a. However, when the injection sites were analyzed using the portable harmonic response measurement system (at modulation frequency 500 Hz), they were clearly distinguishable due to the phase angle differences, as shown in Fig. 5b. It is worthwhile mentioning that the time delay of both the excitation and emission photons caused by tissue scattering (typically in the order of tens of nanoseconds⁵⁷) will not introduce a notable phase lag in our technique, ensuring the applicability of this technique in deep tissues. Importantly, when multiple groups of UCNPs with different phase angle features are present at the same location, there is a potential to quantify their respective contributions by performing measurements at multiple frequencies in a critical frequency range, followed by a proper fitting procedure. This is discussed in detail in Supplementary Section 6.

It should be pointed out that our phase-angle encoding/decoding approach provides a superior alternative to previously reported

lifetime multiplexing techniques. First, the phase angle can be effectively decoded with an inexpensive and miniaturized system and with an easy fitting procedure. Second, the signal-to-noise ratio in the decoding process is supposed to be significantly better than in the time-domain lifetime multiplexing technique, since the excitation offset (average excitation intensity) can remain at high levels to obtain high luminescence intensity, while in the time-domain technique the signal-to-noise ratio is generally low due to weak luminescence signals under short pulse laser excitation of low duty cycles. In addition, the dynamic range of the phase angle for UCNPs (0 - 180°) is essentially larger than that of a sole decay lifetime, which is equivalent to (0 - 90°) by phase angle though it could cover several orders-of-magnitude.

4. Conclusions

Upconversion nanoparticles have complex luminescence mechanisms featuring complicated kinetics, opening possibilities for developing unique optical multiplexing techniques in other domains than the conventional spectral, intensity, spatial dimension, and lifetime domains. In this work, a new optical encoding dimension in the frequency domain – the phase lag of upconversion luminescence in response to harmonic-wave excitation – was created, taking advantage of the rise and decay times governing the luminescence kinetics. We provided theoretical insight for the luminescence kinetics of UCNPs after short pulse excitation and have constructed the connection between the phase lag and the rise and decay time constants. Experimentally, we developed methods for engineering upconversion luminescence kinetics including employing the effects of doping concentration, energy migration, ion quenching, and surface quenching, and eventually achieved nanoparticles with tunable phase angles in a wide range. Such phase-angle encoded UCNPs can be potentially used in many multiplexing applications, e.g., anti-counterfeiting and deep tissue multiplexed labelling/detection as demonstrated in this work. We

believe that our work will greatly increase the multiplexing capacity of UCNP-based techniques and will also shine light on developing new discernible identities to meet the needs of increasing requirements of multiplexed applications.

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