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RECENT ADVANCES IN ULTRASOUND-CONTROLLED FLUORESCENCE TECHNOLOGY FOR DEEP TISSUE OPTICAL IMAGING: A REVIEW

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Abstract:

Fluorescence imaging is a non-invasive and dynamic real-time imaging technique; however, it exhibits poor spatial resolution in centimetre-deep tissues because biological tissues are highly scattering media for optical radiation. The recently developed ultrasound-controlled fluorescence (UCF) imaging is a novel imaging technique that can overcome this bottleneck. Previous studies suggested that the effective contrast agent and sensitive imaging system are the two pivotal factors for generating high-resolution UCF images ex vivo and/or in vivo. Here, this review highlights the recent advances (2015–2020) in the design and synthesis of contrast agents and the improvement of imaging systems to realize high-resolution UCF imaging of deep tissues. The imaging performances of various UCF systems, including the signal-to-noise ratio, imaging resolution, and imaging depth, are specifically discussed. In addition, the challenges and prospects are highlighted. With continuously increasing research interest in this field and emerging multidisciplinary applications, UCF imaging with higher spatial resolution and larger imaging depth may be developed shortly, which is expected to have a far-reaching impact on disease surveillance and/or therapy.

Keywords : *Ultrasound-controlled fluorescence imaging, Temperature-sensitive NIR probes, High-resolution, Deep tissue, Molecular diagnosis.*

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INTRODUCTION:

Fluorescence imaging has gained significant attention as a non-invasive tool in the basic study and clinical applications. It is a highly convenient and effective tool for real-time tracing of various fluorescence signals in biological applications because it can endow a high signal-to-noise ratio (SNR) and commendable spatial resolution during the imaging process. In general, when light passes through biological tissue, the following light-tissue interactions can occur: photon reflection, photon scattering, photon absorption, and tissue autofluorescence. The main obstacles of fluorescence imaging include tissue autofluorescence, fluorescence quenching, photo bleaching of the fluorescence photons, and poor tissue penetration. Typically, fluorescence imaging is realized in the wavelength scopes from 400 nm to 700 nm (i.e., visible window) and from 700 nm to 1700 nm (i.e., near-infrared ray (NIR) window). However, NIR-fluorescence imaging offers more advantages, such as lower photon scattering and minimal autofluorescence interference. More specifically, four biological windows are considered in the NIR region: NIR-I (700–900 nm), NIR-II (1000–1700 nm), NIR-III (1700–1870 nm), and NIR-IV (2100–2300 nm) windows. NIR fluorescence imaging with high resolution and satisfactory SNR exhibits immense potential in molecular detection and treatment applications [1].

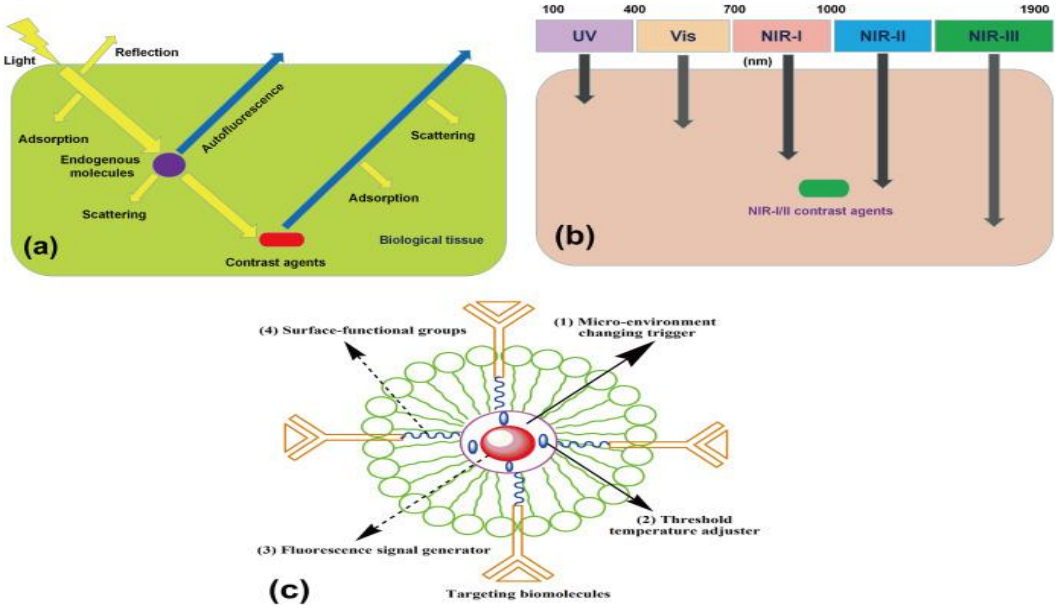

FIG 1: (A) Schematic illustration of the light and the tissue (B) The schematic diagram of depth of penetration for the NIR-I/II light. (C) The UCF contrast agent's components. UCF: ultrasound-controlled fluorescence.

Compared with classical magnetic resonance imaging, computed tomography (CT), and positron emission tomography, NIR fluorescence imaging is a more effective, inexpensive, and ultrasensitive approach for real-time molecular imaging. Presently, the poor resolution and insufficient penetration depth in deep tissues are the primary factors that limit the universal clinical use of the NIR fluorescence technique. A particular issue is that the spatial resolution of the NIR fluorescence imaging sharply decreases with increasing tissue thickness. In order to resolve this issue and obtain good spatial resolution for fluorescence imaging of deep tissues, several potential approaches were proposed. For instance, a broadband lock-in amplifier and fluorescent microbubbles for detecting the signals of ultrasound-controlled fluorescence , ultrasound-adjusted luminescence imaging without an external excitation within a scattering medium , ultrasound-controlled fluorescence imaging based on temperature-responsive fluorescence probes , the ICG-loaded Pluronic nanocapsules as both fluorescence intensity and lifetime probe for generating the ultrasound-controlled fluorescence images , combined the light-pulse-delay and photon counting methods for obtaining the ultrasound-controlled fluorescence

imaging by using a DBD-AA (i.e., N-{2-[(7 –N, N-dimethylaminosuflonyl)-2,1, 3-benzoxadiazol-4-yl] (methyl)amino} ethyl-N-methylacrylamide) labelled poly N-isopropylacrylamide probe , ultrasound-mediation of fluorescence imaging within a scattering medium based on the bioluminescence and chemiluminescence mechanisms , and time-reversed ultrasonically encoded optical focusing toward transmit light into any defined location within a scattering medium . Nevertheless, the limited photons in the detected area restrict the acceptable sensitivity of NIR fluorescence imaging. The ultrasound-modulated tactics can realize good acoustic resolution in deep scattering media during the fluorescence imaging. However, manipulating the on/off signals of the NIR fluorescence imaging probe in living tissues via an ultrasonic modulator remains a significant challenge.

This review provides a detailed summary of the advances (2015–2020) in UCF imaging for deep tissue phantoms and in vivo imaging and offers a systematic approach that can be used to improve the UCF imaging system. Here, we briefly review the synthetic methods for UCF contrast agents and the emission characteristics, morphology, and surface characteristics of ICG-based UCF probes. The emerging applications of ICG-based UCF probes, including temperature-sensitive polymer nanoparticles (NPs) and thermosensitive liposomes, in the field of deep-tissue NIR fluorescence imaging, are also summarized. In addition, the advances of the UCF imaging system in the last five years are summarized. Finally, new opportunities and future prospects for developing UCF imaging probes and UCF systems are discussed [2].

NIR-UCF imaging vs. traditional NIR imaging:

Fluorescence imaging as a potential diagnostic tool displays good temporal-spatial resolution and high sensitivity; however, it faces the challenge of poor tissue penetration in the visible window (400–700 nm). In contrast, fluorescence imaging in the wavelength scope of 700–900 nm has some advantages, such as minimal tissue absorption, background fluorescence, and deeper tissue penetration. Therefore, although NIR fluorescence imaging has received extensive attention as a non-invasive imaging technique in the past two decades, its practical applications are still restricted by limited tissue penetration and low spatial resolution. The NIR-II fluorescence imaging has recently shown ameliorations in spatial resolution and imaging depth. In particular, NIR-II fluorescent imaging can be used to probe centimetre-deep tissues and realize micrometre-scale resolution at depths of millimetres (Fig:2). However, all reported NIR-II fluorophores are slowly excreted and are principally retained within the reticuloendothelial system, making clinical translation/application significantly difficult Compared with the traditional NIR fluorescence imaging, the UCF imaging technique has exhibited the significant potential to provide high-resolution fluorescence images for biomedical exploration. Specifically, the ultrasound-adjusted fluorescence strategy was first reported in 2006. Later, two independent research groups reported the utilization of the UCF imaging method in 2012. These ground-breaking developments facilitated an exciting future for high-resolution deep-tissue imaging based on fluorescence. In particular, Yuan and co-workers recently demonstrated that the poly (N-isopropylacrylamide) (PNIPAM)/β-cyclodextrin(β-CD)/ICG Nano gels as a potential UCF probe can be detected up to ∼3.5 cm in the chicken breast tissue, which was better than the depths of previously reported NIR-I and NIR-II [contrast agents and close to the imaging depths of ∼3.2 cm and ∼3.0 cm in pork tissue and slice pork ham using the NIR-I core/shell nanoparticles and NIR chemiluminescence emitter , respectively. The earlier studies have also suggested that NIR-UCF imaging provides better SNR and spatial resolution as well as more comprehensive information (such as three-dimensional (3D) fluorescence images) on the deep tissues than conventional NIR-I technique, which can boost the synthesis of new NIR-UCF contrast agents within the NIR-I and NIR-II regions and further promote the biomedical applications of the NIR-UCF imaging.

Classification of NIR-UCF contrast agents:

1. **Composition and working mechanism of UCF contrast agents:**

A superior UCF contrast agent plays an important role in deep tissue imaging with a high-resolution level. The UCF contrast agent generally contains four key components (Fig. 3): A trigger that changes the micro-environment of the UCF contrast agent. The temperature-sensitive polymer NPs or micelles such as PNIPAM or Pluronic polymer have been used for achieving this goal because they exhibit excellent temperature-triggered phase transition. An adjuster that tunes the threshold temperature of the UCF contrast agent. The PNIPAM or Pluronic polymer usually conjugates with a hydrophilic or hydrophobic compound to adjust the threshold temperature of the polymer. (3) A fluorescence emitter that generates the amplified fluorescence signal via increasing the temperature. A microenvironment-sensitive emitter, such as a polarity-sensitive fluorophore, is used for generating the “ON” fluorescence emission signal.

(4) The surface-functional groups containing $–NH_2$, –COOH, and –OH groups can interact with the targeting biomolecules , which facilitates the active targeting and aggregation efficiency of contrast agents to the lesion tissues.

The working mechanism of the UCF contrast agent includes the following steps: (1) The heat energy is firstly produced by high-intensity focused ultrasound (HIFU) technique; then, the confined temperature of the UCF probe is increased. The internal micro-environment (i.e., hydrophilic characteristic) of the UCF probe is changed through the HIFU heating, which leads to a phase transition and weakening of polarity. Thus, the hydrophobic property of the UCF probe becomes stronger. The quantum yield of the fluorophore increases with decreasing polarity, causing an enhancement in the absolute fluorescence intensity or fluorescence lifetime. That is, the emitted fluorescence from UCF probes is confined within the focused ultrasound region during UCF imaging. In contrast, weak or no fluorescence is produced when the UCF probes are located outside the focal volume. Therefore, the detected signal is observed as “ON” within the ultrasound confined volume because the background fluorescence in the “OFF” status is negligible without HIFU treatment. This also implies that the spatial resolution of UCF imaging primarily relies on the size of confined thermal space. Besides, the outgoing fluorescence signal can be readily regulated by changing the ultrasound parameters and can only be generated from the confined UCF probes [3].

2. Temperature-sensitive polymer nanoparticles: As a representative contrast agent, the ICG-PNIPAM NPs , have been used for NIR-UCF imaging . The ICG-PNIPAM NPs with a spherical structure were initially prepared by free-radical polymerization in an aqueous medium where sodium dodecyl sulfate (SDS), ammonium persulfate (APS), and tetramethylethylenediamine (TMEDA) served as the surfactant, initiator, and accelerator, respectively . ICG aqueous solution was premixed with the said polymer aqueous solution; ICG molecules with amphiphilic property were then encapsulated inside the SDS-based micelles. Finally, the ICG-PNIPAM NPs were obtained via a free-radical emulsion polymerization method under an inert atmosphere. In addition, the threshold temperature (either lower or higher) of the ICG-PNIPAM NPs could be tuned by conjugating with hydrophobic N-tert-butylacrylamide or hydrophilic acrylamide. The storage time of ICG-PNIPAM NPs is below 30 days. In particular, the ICG dye can be easily oxidized by APS or TMEDA during the free-radical polymerization process. In order to overcome this limitation, the 4-4′-azobis (4-cyanopentanoic acid) (ACA) was used to replace APS and TMEDA as the initiator and catalyst, and synthesize the ICG-PNIPAM NPs . The results indicated that the modified ICG-PNIPAM NPs had a good shelf life of 6 months. Meanwhile, considering the toxicity of the surfactant SDS, Yu et al. also explored the Pluronic F-127 or F-98 to replace SDS for fabricating the ICG-PNIPAM NPs. The toxicity of the ICG-PNIPAM NPs using Pluronic F-127 or F-98 as a surfactant is still unclear.

In general, in vitro and in vivo testing are essential for evaluating the imaging performance of any contrast agent. For in vitro or ex vivo studies, Yu et al. embedded a silicone tube (inner diameter = 0.76 mm) in a silicone phantom at a depth of ∼5 mm to simulate a blood vessel. They inserted a small silicone tube (inner diameter = 0.31 mm) into a piece of porcine muscle tissue (thickness = 10 mm) to simulate a real tissue microenvironment. For both the above models, high quality ex vivo UCF images with good SNR were achieved by using ICG-PNIPAM NPs with ACA as the initiator. However, in vivo UCF imaging has not been realized using the same probe. Later, Yao et al. obtained that the ICG-PNIPAM NPs were moderately stable in the biological environment; they were mainly accumulated into the spleen of the mice. Meanwhile, the in vivo UCF imaging of mouse breast tumors and in vivo/ex vivo UCF imaging of the mouse spleen were successfully realized using ICG-PNIPAM NPs and frequency-domain (FD)-UCF system. The main disadvantages of this contrast agent are that the size is too large and the toxicity is unclear, especially the targeted property is lacking.

For elucidating the above-mentioned results, the two-dimensional (2D) fluorescence planar image of the locally injected ICG-PNIPAM NPs in a mouse's breast tumor and the shell temperature of the mouse are shown in Figures.

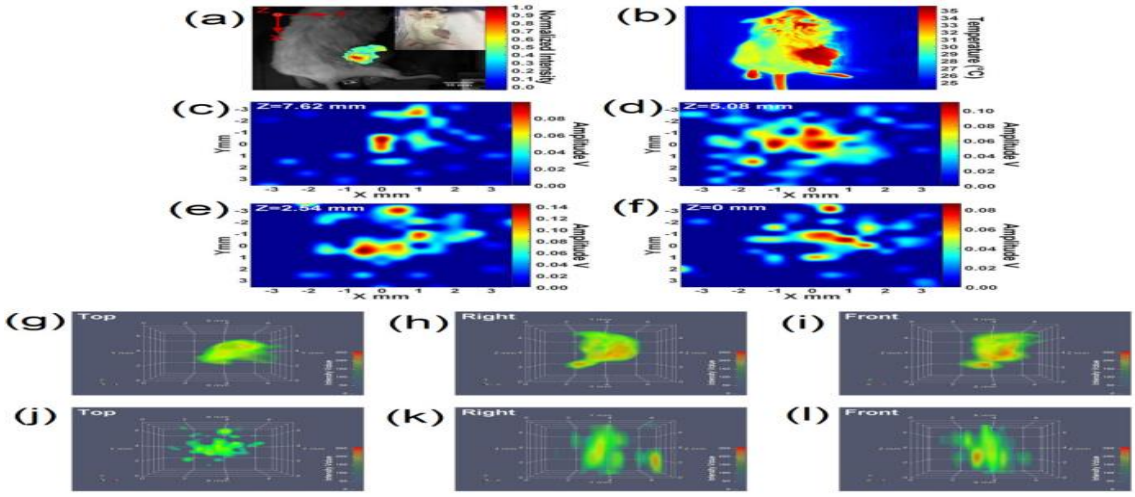

Fig 2 : The fluorescence image of the mixed contrast agents via local injection in the tumor (Ex/Em = 808/830 nm).

The top-right inset is the photograph of the mouse with a breast tumor. The red square is represented as UCF scan area. (B) A thermal imaging image of the mouse. (C–F) 2D-UCF images of different depths at X–Y plane. (G–I) The top, right and front side views of the 3D CT image. (J–L) The top, right and front side views of the 3D UCF image. 2D: two-dimensional; 3D: three-dimensional.

Thermosensitive liposomes:

Liposomes have a single or multiple phospholipid bilayer membrane outside the internal aqueous core. They can be used to encapsulate hydrophilic drugs within the aqueous core, whereas lipophilic drugs can be incorporated into the membrane. The size of the spherical vesicle is between micrometers and nanometers. In general, liposomes within the size range of 50–450 nm are employed in nanomedicine. The size of the liposome is a key factor for clinical use. The relevant findings indicate that the large-sized liposomes are quickly removed from the body[4].

Although the ICG-liposome has been successfully used for ex vivo and in vivo NIR-UCF imaging, synthesis of the small-sized or nanoscale ICG-liposome with specific targeting functions still needs to be explored in the future. A smaller liposome with a larger surface area is s significantly beneficial to bypass biological barriers for blood vessel circulation. In particular, the nanoscale ICG-liposome with active targeting characteristics for UCF imaging is highly desired in the future, which will significantly help in improving the sensitivity and temporal-spatial resolution of in vivo NIR-UCF imaging.

Overall, the success of in vivo UCF imaging primarily depends on the physiological stability of probes in biological tissues. In addition, the UCF probe with an excellent switchable property can ameliorate the SNR and reduce the required ultrasound power. Besides, an advanced and ultrasensitive UCF imaging system is also essential to generate high-quality UCF images. Such a UCF imaging system that can provide comprehensive 3D information is conducive to future biomedical research [5].

Advances in NIR-UCF imaging systems:

In general, based on the variation in the fluorescence signal caused by ultrasound, the UCF imaging pattern is divided into two kinds: fluorescence intensity-based UCF and fluorescence lifetime-based UCF imaging. The existing studies show that high-resolution imaging of deep tissues can be achieved using both these imaging modalities. Herein, these imaging modalities are briefly reviewed.

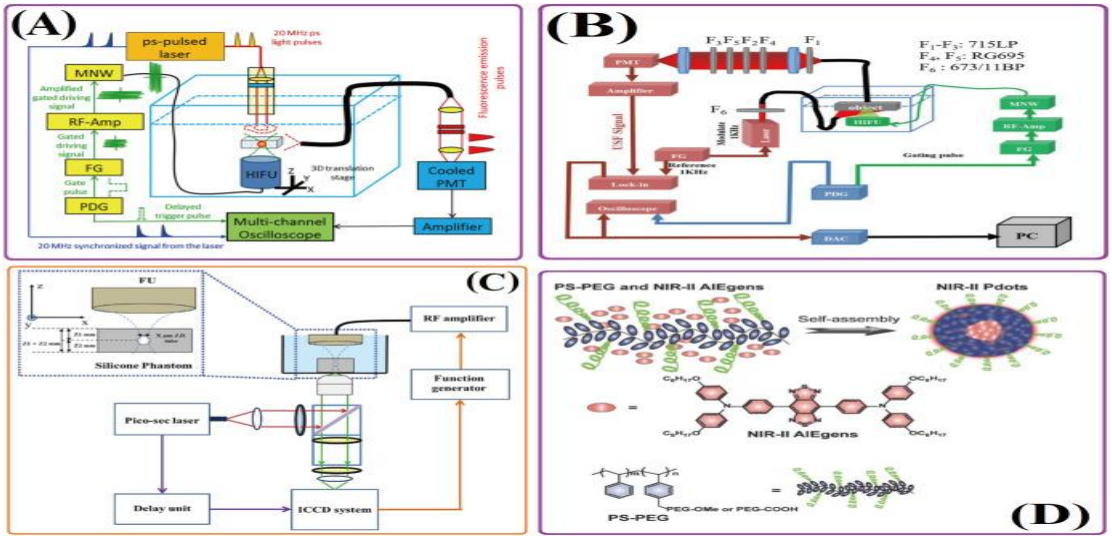

Fig. 4. (A) Illustration of the fluorescence lifetime-based UCF imaging system. (B) Schematic diagram of the frequency-domain UCF imaging system with a fluorescence intensity readout. (C) Illustration of ICCD camera-based time-domain UCF system. (D) Schematic of the preparation of NIR-II polymer dots. EMCCD: electron multiplying charge coupled device; ICCD: intensified charge coupled device [6].

1. Fluorescence intensity-based UCF imaging system

The working principle of the fluorescence intensity-based UCF imaging system is described as follows: first, the UCF contrast agents are injected into a silicone tubing within the biological tissue sample. An 808-nm semiconductor laser is used for continuously irradiating the superstratum of the tissue sample. The fluorescence signal of the NIR-UCF probes is generated under laser irradiation and passed through the biological tissue to the surface of the sample. Then, it is collected by a fiber bundle. The acquired fluorescence signal is transmitted through a specific filter and is further converted into the electrical signal via a photomultiplier tube (PMT); the electrical signal is displayed on an oscilloscope. The detected area of the sample is scanned by a focused ultrasound pulse in a point-by-point way. During the operation, the intensity and duration of the ultrasound pulse and the collection of fluorescence signals are manipulated by a pulse delay generator. The UCF images are finally reconstructed by the signals at each pixel. According to the above working mechanism, the resolution of UCF imaging is mainly influenced by the focal spot size of the focused ultrasound pulse. The imaging depth significantly depends on the switching multiple of the fluorescence signal and the SNR and sensitivity of the UCF imaging system.

The fluorescence intensity-based UCF imaging system was ameliorated by the same research group in 2016 . Based on the previous UCF imaging system, they mainly implemented the following improvements.The 808-nm semiconductor laser could be used to modulate the intensity of excitation light; the generated fluorescence signal was collected by using the phase-locked amplification technique. The fluorescence signal was correlated with the frequency domain characteristics for differentiating the UCF signal. The fluorescence intensity of the UCF probes could be enhanced more than 200 times by using focused ultrasound. The results indicated that a resolution of 900 μm was obtained in the 3.1 cm-thick biological tissue phantoms [7].

2. Fluorescence lifetime-based UCF imaging system:

The enlargement of the fluorescence lifetime for the UCF probes under the HIFU radiation was initially reported by Yuan et al. in 2012, a picosecond pulsed laser as the excitation light source was used to irradiate the sample in this imaging system. Typically, the temperature of the sample at the focus area of the acoustic field increased due to the ultrasonic radiation, leading to an increase in the fluorescence lifetime of the UCF probe. Thus, it was highly possible to identify the UCF signal by detecting the fluorescent photons after a specific delay time of the excitation light pulse. We note that the ability of this system to execute sub-millimeter-resolution deep-tissue imaging was proved in this work. The SNR of the fluorescence lifetime-based UCF imaging is required to be higher than that of the

fluorescence intensity-based UCF imaging, which is more attractive for high-quality UCF imaging. However, the relevant reports on this topic are rare. Therefore, novel NIR-UCF imaging techniques based on the fluorescence lifetime principle is still highly desirable for the monitoring and therapy of diseases [8,9].

Although various UCF imaging systems have been developed in the last few years, each imaging system has its advantages and disadvantages. The newly developed EMCCD-gain-controlled UCF imaging system with high resolution, large scan speed, excellent SNR, and better imaging depth exhibits immense potential for in vivo UCF imaging and investigation on deep-tissue lesions. It will be useful for examining the vascular dynamics in animal models and understanding the cerebral diseases in deep tissues. From the perspective of ultrasonic control conditions, the optimized parameters of the given HIFU conditions are also useful for improving the performance of the ex vivo or/and in vivo UCF imaging [10].

CONCLUSIONS:

Over the past few years, the UCF technique has been established as a novel tool to realize high-resolution fluorescence imaging in centimetre-deep tissues. One of its significant attributes is that it resolves poor spatial resolution in the deep tissues. Besides, a histological 3D image of the entire organ can be obtained by this imaging tool, which may provide useful insights on the tissue morphology, physiological processes, and the invasion and metastasis of tumors. Several ICG-based UCF probes have been excavated and used for ex vivo and/or in vivo NIR-UCF imaging. The thriving nanotechnology field can boost the design and synthesis of new UCF contrast agents. It has significant potential to exploit novel disease diagnostic methods in centimetre-deep tissues. In the present review, the recent advances (2015–2020) in the design and synthesis of the contrast agents and the improvements in the imaging systems for realizing high-resolution UCF imaging of deep tissues were summarized. In particular, recent progress in the evolution of the UCF probes, improvements in the UCF imaging systems, and in vivo UCF imaging were systematically explored.

Some key developments occurred in this field. However, several challenges still limit the practical applications of in vivo UCF imaging technology:

(1) limited polarity/temperature-sensitive NIR dyes and stable thermo-responsive polymer carriers, especially lack of the active targeting function
(2) poor stability and retention time of the UCF probes in vivo
(3) low on/off ratio of the UCF contrast agents
(4) potential toxicity of the developed UCF probes
(5) absence of highly sensitive and fast scanning UCF imaging systems
(6) optimization of such parameters, including the ultrasound control conditions and scanning model, might solve the current problems.

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