A Two-in-One Janus NIR-II AIEgen with Balanced Absorption and Emission for Image-Guided Precision Surgery

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Abstract

Fluorescence imaging in the near-infrared II (NIR-II, 1000-1700 nm) region opens up new avenues for biological systems due to suppressed scattering and low autofluorescence at longer-wavelength photons. Nonetheless, the development of organic NIR-II fluorophores is still limited mainly due to the shortage of efficient molecular design strategy. Herein, we propose an approach of designing Janus NIR-II fluorophores by introducing electronic donors with distinct properties into one molecule. As a proof-of-concept, fluorescent dye 2TT-\textit{m},oC6B with both twisted and planar electronic donors displayed balanced absorption and emission which were absent in its parent compound. The key design strategy for Janus molecule is that it combines the merits of intense absorption from planar architecture and high fluorescence quantum yield from twisted motif. The resulting 2TT-\textit{m},oC6B nanoparticles exhibit a high molar absorptivity of $1.12 \times 10^4$ M$^{-1}$ cm$^{-1}$ at 808 nm and a NIR-II quantum yield of 3.7%, displaying a typical aggregation-induced emission (AIE) attribute. The highly bright and stable 2TT-\textit{m},oC6B nanoparticles assured NIR-II image-guided cancer surgery to resect submillimeter tumor nodules. The present study may inspire further development of molecular design philosophy for highly bright NIR-II fluorophores for biomedical applications.

Introduction

Cancer is responsible as the leading cause of death globally.1 Tumor detection at an early stage is crucial for a successful treatment. Although advances have been made in traditional diagnostic methodologies, such as magnetic resonance imaging, computed tomography and positron emission tomography, they do
not attain the required sensitivity and specificity for detecting the disease at an early-stage.\textsuperscript{2,3} Fluorescence imaging-guided cancer surgery has been demonstrated clinically to detect tiny tumor foci or the exact borders between normal and tumor tissues during surgery owing to its high sensitivity, real-time capability, absence of ionizing radiation and portability.\textsuperscript{4,5} Fluorophores emitting in the near-infrared II (NIR-II, 1000-1700 nm) region have received increasing attention because of its merits for having increased tissue penetration, improved spatial resolution and high signal-to-background ratio.\textsuperscript{6-15} To date, outstanding achievements have been obtained by inorganic materials, attaining enhanced brightness for biomedical applications.\textsuperscript{16-20} However, these inorganic materials generally suffer from potentially long-term toxicity. Alternatively, organic materials with the advantages of good biocompatibility, potential biodegradability and excellent processibility are promising for NIR-II bioimaging.\textsuperscript{21-27} However, the molecular design philosophy for organic NIR-II fluorophores is not widely reported, as traditional methods rely on trial and error via extensive tuning of chemical structures with promising motifs. Thereof, a convenient yet efficient molecular design strategy for highly efficient NIR-II fluorophores is fascinating.

It is widely accepted that increasing the length of $\pi$-conjugation and engineering donor-acceptor (D-A) units can efficiently shift the fluorescence emission towards the long-wavelength region.\textsuperscript{28-31} These molecules are typically featured with planar architectures to exhibit strong absorption and emission signals as isolated species. However, when they are dispersed in biological medium (water), the resultant intense intermolecular $\pi$-$\pi$ interactions often quench the fluorescence.\textsuperscript{32} Such aggregation-caused quenching (ACQ) effects make them unsuitable for bioimaging applications, which may give false feedback on tumor diagnosis. As a technology related to aggregate-state luminescence, aggregation-induced emission (AIE) holds the potential to tackle this issue.\textsuperscript{33-38} AIE luminogens (AIEgens) commonly own twisted structures that can efficiently suppress intermolecular interactions, resulting in improved fluorescence in the aggregated state.\textsuperscript{39,40} However, the twisted architecture blue-shifts the absorption peak, which hinders the laser excitation for deep tissue imaging in biological applications. Therefore, it remains challenging to develop NIR-II fluorophores with maximum absorption at longer wavelengths with high fluorescence quantum yield (QY).
Herein, we propose a molecular design strategy of developing Janus NIR-II fluorophores through tethering electronic donors with distinct properties into acceptors. As a proof-of-concept, as shown in Fig. 1, by combining the planar donor from ACQ-dominated 2TT-\(m\)C6B and twisted donor from AIE-active 2TT-\(o\)C6B, the newly formed molecule 2TT-\(m,o\)C6B possessed both planar and twisted donors. As a consequence of such molecular design, 2TT-\(m,o\)C6B displayed a typical AIE attribute due to the restriction of intermolecular interactions by the twisted donor. Compared to 2TT-\(o\)C6B, 2TT-\(m,o\)C6B showed a 54 nm redshift in absorption peak (750 nm) with an emission peaked at 1059 nm. Meanwhile, the QY of 2TT-\(m,o\)C6B aggregate reached up to 3.7%, which was better than 2TT-\(m\)C6B (0.75%). As a result, the 2TT-\(m,o\)C6B nanoparticles (NPs) are promising for NIR-II fluorescence imaging, which outperforms the clinically approved dye. Given the high brightness, the 2TT-\(m,o\)C6B NPs can act as an effective fluorescent agent for tumor imaging-guided surgery to resect tiny tumor modules that are indistinguishable to surgeons. The present study may inspire some insights into the molecular design philosophy of highly bright NIR-II dyes for biomedical applications.

**Results and discussion**

**Molecular synthesis and design**

The synthetic routes and characterization of the three molecules associated with intermediates were shown in the supplementary material [Scheme (1-3), Figures (1-16)]. Traditionally, most NIR-II fluorophores are derived from D-A-D structures. In these molecules, benzobisthiadiazole (BBTD) is often employed as...
strong acceptors, whose stabilized quinoidal structure can red-shift the emission to the NIR-II region.\textsuperscript{41-43} Electron donors play decisive roles in the fluorescence properties of D-A-D fluorophores. For instance, 4-(2-thienyl)triphenylamine (TTA) moiety has been demonstrated as both electronic donor and π-conjugated bridge, which can couple with BBTD to afford long-wavelength emission.\textsuperscript{43,44} Through the introduction of alkyl chains to different positions of thiophene (T) unit, the resulting fluorophores can show distinct photophysical properties. As shown in Fig. 1, molecule 2TT-\textit{m}C6B with a hexyl unit located on the 3-position of T displayed a coplanar T-BBTD-T structure (3-TBT, dihedral angle of 1°), owing to its useful conjugation.

On the one hand, the planar architecture gave 2TT-\textit{m}C6B intense absorption in the long-wavelength range, which could not only penetrate deep tissues but also reduce photo-damage to the living body.\textsuperscript{43} On the other hand, the planar structure also resulted in strong intermolecular π-π interactions in the aggregated state, giving undesirable ACQ effect. Alternatively, molecule 2TT-\textit{o}C6B with a hexyl graft on the 4-position of T displayed a distorted T-BBTD-T structure (4-TBT, dihedral angle of 48°), owing to the enhanced steric hindrance between BBTD core and alkyl chains.\textsuperscript{29} The twisted backbone coupled with triphenylamine (TPA) rotor endowed 2TT-\textit{o}C6B AIE attribute by efficient restriction of intramolecular interactions in the aggregated state. Notably, AIE-active 2TT-\textit{o}C6B showed a high solid-state QY of 8.4\%, which was beneficial for bioimaging.\textsuperscript{29} However, the distorted architecture broke the conjugation, resulting in an unsatisfactory blue-shift in absorption. Collectively, these two molecular design strategies possessed advantages and disadvantages in fluorescence imaging. Can we integrate long-wavelength absorption and high QY into one molecule to attain a highly bright NIR-II fluorophore? To address this issue, we proposed a molecular design strategy of incorporating different donors into one molecule (denoted “Janus” molecule). The key design strategy for Janus molecule was to combine planar and twisted TTA moieties into BBTD core [Fig. 1]. The planar unit was expected to afford strong absorption in long-wavelength range, while the twisted counterpart was hoped to supply high QY in the aggregate state. The resultant two-in-one molecule 2TT-\textit{m,\textit{o}}C6B with balanced absorption and emission property may improve imaging quality in biological applications.

\textbf{Theoretical calculation}
FIG. 2. Theoretical calculation data of 2TT-mC6B, 2TT-m,oC6B and 2TT-oC6B. (a) Chemical structures. (b) Molecular architectures and dihedral angles of the optimized S₀ geometries. (c) Calculated HOMOs and LUMOs of the molecules. The HOMO and LUMO energy levels and their gaps are also displayed.

In order to confirm the molecular conformation [Fig. 2(a)], density functional theory (DFT) calculations were performed using Gaussian 09 software. The dihedral angle between T and BBTD was 1° in the optimized ground-state (S₀) of 2TT-mC6B, while it was 48° in 2TT-oC6B [Fig. 2(b)]. These results confirmed the important roles of alkyl chains in determining the molecular architectures. Notably, the dihedral angles in Janus molecule 2TT-m,oC6B were 5° and 48° for 3-TBT and 4-TBT moieties, respectively, suggesting the adaption of a planar plus twisted architecture. This result demonstrated the feasibility of the present molecular design strategy. Figure 2(c) shows the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) of the molecules. The molecular HOMO was delocalized along the whole molecular backbone for the three dyes, while the LUMO location was different. For example, the LUMO in 2TT-mC6B was localized on both central T-BBTD-T core and part of the TPA unit owing to the better conjugation. However, it was only located at the T-BBTD-T core.
for 2TT-\(o\)C6B because of the breakage in conjugation. Interestingly, the LUMO of 2TT-\(m, o\)C6B was located at both BBTD-T-TPA and BBTD-T units, precisely inheriting from its parents. Moreover, the energy gap between HOMO and LUMO in 2TT-\(m, o\)C6B was 1.35eV, which was between 2TT-\(m\)C6B (1.24 eV) and 2TT-\(o\)C6B (1.47 eV). This narrow energy gap was favorable for strong absorption in the NIR biological region. Overall, all these data supported the success of the present strategy, demonstrating an accurate manipulation of the photophysical properties by structural modification.

**Photophysical properties**

Given the novel planar/twisted structure of 2TT-\(m, o\)C6B, the photophysical properties were compared with its parent compounds. As shown in Fig. 3(a), the absorption maximum of 2TT-\(m, o\)C6B (750 nm) in tetrahydrofuran (THF) outperformed that of 2TT-\(o\)C6B (696 nm), indicating that introduction of the planar unit was beneficial for red-shifting the absorption.\(^{28}\) Meanwhile, the blueshift of absorption maximum in contrast to 2TT-\(m\)C6B (816 nm) suggested the existence of twisted structure in 2TT-\(m, o\)C6B. Besides the absorption wavelength, molar extinction coefficient (\(\varepsilon\)) is also a key factor to determine the penetration depth. Based on the widespread use of 808 nm laser in biological applications, the \(\varepsilon\) at this wavelength was investigated. The \(\varepsilon\) of 2TT-\(m, o\)C6B (1.06 \(\times\) 10\(^4\) M\(^{-1}\) cm\(^{-1}\)) at 808 nm was significantly higher than that of 2TT-\(o\)C6B (0.53 \(\times\) 10\(^4\) M\(^{-1}\) cm\(^{-1}\)), even though slightly lower than that of 2TT-\(m\)C6B (1.4 \(\times\) 10\(^4\) M\(^{-1}\) cm\(^{-1}\)). These data demonstrated the positive effect of planar architecture on the absorption efficiency. Then the photoluminescence (PL) property of the dyes was studied. As shown in Fig. 3(b), their emission maxima in THF belonged to the NIR-II region, which was beneficial for high clarity fluorescence imaging. Notably, compared to the unimodal profile of 2TT-\(o\)C6B (1013 nm) and 2TT-\(m\)C6B (1070 nm), 2TT-\(m, o\)C6B displayed multimodal fluorescence spectrum ranging from 1000 to 1100 nm owing to the mixed planar and twisted structure. Since the dye molecules are normally in the form of aggregated state in life medium, their photophysical properties are thus investigated. Upon aggregation, a redshift in absorption maxima compared to the solution profile was observed for the three molecules owing to the intermolecular interactions [Fig. 3(c)].\(^{35}\) For example, the 2TT-\(m, o\)C6B aggregate exhibited an absorption peak at 774 nm, a 24 nm redshift in comparison with the solution spectrum. Accompanied with the redshift of the absorption peak, the \(\varepsilon\) at 808 nm slightly increased to 1.12 \(\times\) 10\(^4\) M\(^{-1}\) cm\(^{-1}\) for 2TT-\(m, o\)C6B. Notably, 2TT-\(m, o\)C6B exhibited strong PL signal in the aggregated state with an emission peak at \(~\)1050 nm [Fig. 3(d)].
FIG. 3. (a) Molar absorption coefficient of molecules in THF. (b) PL spectra of molecules in THF. (c) Molar absorption coefficient of molecules in aggregate ($f_w = 90$ vol%), $f_w$ refers to the water volume fraction in water/THF mixture. (d) PL spectra of molecules in aggregate ($f_w = 90$ vol%). (e) PL spectra of 2TT-$m,o$C6B in THF/H2O mixture with different $f_w$. (f) Variation of PL intensity of the molecules with $f_w$.

To confirm the AIE or ACQ properties of 2TT-$m,o$C6B, the fluorescence intensity fluctuations of the molecules in THF/water mixtures with different water volume fractions ($f_w$) were investigated. As shown in Fig. 3(e), the fluorescence signal was progressively weakened with the increase of $f_w$ from 0% to 40%, due to the forming of twisted intramolecular charge transfer (TICT) state.$^{46,47}$ The TICT state goes back to the ground state mainly through nonradiative decay, thus resulting in a decrease in fluorescence intensity.$^{48-50}$ Further increasing $f_w$ to 90% gradually intensified the PL signal. These data demonstrated the typical AIE attribute of 2TT-$m,o$C6B, as a result of the restriction of intermolecular interactions by the half-twisted
structure in the aggregated state (Figure 17 of the supplementary material). \( \alpha_{\text{AIE}} \) (PL intensity at \( f_w = 90\% \) versus \( f_w = 0\% \)) is a factor that can distinguish the emission efficiency in the aggregated state. The \( \alpha_{\text{AIE}} \) of 2TT-\( m,o\)C6B (1.13) was in between 2TT-\( o\)C6B (6.12) and 2TT-\( m\)C6B (0.06), suggesting its unique twisted/planar architecture. All these results are supportive that 2TT-\( m,o\)C6B with balanced absorption and emission could be promising for NIR-II fluorescence imaging.

**FIG. 4.** Photophysical properties of 2TT-\( m,o\)C6B NPs. (a) Fabrication of 2TT-\( m,o\)C6B NPs using amphiphilic polymer DSPE-PEG\(_{2000}\). (b) Normalized absorption and emission profile of 2TT-\( m,o\)C6B NPs. (c) The plots for the integrated fluorescence spectra (1000–1500 nm) of the sample nanoparticles and IR-26 (1000–1500 nm) at five different concentrations. (d) The colloidal stability of 2TT-\( m,o\)C6B NPs in \( \text{H}_2\text{O} \), PBS and FBS, respectively. (e) Photostability of the 2TT-\( m,o\)C6B NPs versus ICG upon 808 nm laser
irradiation (0.8 W/cm²). (f) The relationship between fluorescence intensity and concentration for 2TT-\textit{m},\textit{o}C6B NPs and ICG, respectively. (g) Comparison of brightness of 2TT-\textit{m},\textit{o}C6B NPs and ICG (100 µg/mL) under different LP filters (1000 nm, 50 ms; 1100 nm, 100 ms; 1250 nm, 200 ms). (h) Comparison of penetration depth of 2TT-\textit{m},\textit{o}C6B NPs and ICG (100 µg/mL, 1250 nm LP filter, 800 ms) under different thickness of chicken tissue.

**In vitro imaging**

In order to support 2TT-\textit{m},\textit{o}C6B as an effective bio-agent, we fabricated water-soluble NPs through the nanoprecipitation method using biocompatible amphiphilic polymer DSPE-PEG\textsubscript{2000} [Fig. 4(a)]. Fabrication of NPs with amphiphilic polymers is extremely valuable for biological applications, as it displays excellent in vivo stability, biocompatibility, improved blood circulation time, and passive tumor-targeting ability.\textsuperscript{51,52} Moreover, the resultant NPs triggers the fluorescence mechanism of AIE (restriction of intramolecular motion) to attain a strong fluorescence signal.\textsuperscript{53} As shown in Fig. 4(b), the 2TT-\textit{m},\textit{o}C6B NPs (diameter of \sim 100 nm, Figure 18 of the supplementary material) showed intense absorption in the region of 600-900 nm with a peak at 777 nm, a 27 nm redshift compared to the solution-state spectrum. The 2TT-\textit{m},\textit{o}C6B NPs exhibited PL emission maximum at 1059 nm with a tail extending to 1400 nm, which is capable of NIR-II imaging. The fluorescence QY of 2TT-\textit{m},\textit{o}C6B NPs reached up to 3.7%, which was in the middle of 2TT-\textit{m}C6B (0.75%) and 2TT-\textit{o}C6B (8.4%) [Fig. 4(c)]. This result indicated that we could precisely tune the photophysical properties of the dyes through structural modification. Moreover, 2TT-\textit{m},\textit{o}C6B NPs displayed excellent colloidal stability in different medium, such as water, phosphate-buffered saline (PBS) and fetal bovine serum (FBS) [Fig. 4(d)]. It is crucially important for fluorescence dyes to maintain a reliable signal under different conditions. Thereof, we investigated the photostability of 2TT-\textit{m},\textit{o}C6B NPs, while using the clinically-approved indocyanine green (ICG) as a control. As shown in Fig. 4(e), the PL intensity remained almost unchanged upon continuous laser irradiation (808 nm laser, 0.8 W/cm²), while ICG displayed a rapid decrease in PL intensity with irradiation time. Moreover, the PL intensity of 2TT-\textit{m},\textit{o}C6B NPs increased with the concentration, providing the potential platform for quantitative analysis [Fig. 4(f)]. In contrast, the fluorescence intensity of ICG was slightly increased at low concentration but decreased at high concentration, displaying an ACQ effect. Furthermore, the in vitro NIR-II fluorescence signals of 2TT-\textit{m},\textit{o}C6B NPs were evaluated. Under different long-pass (LP) filters (1000-1250 nm), 2TT-\textit{m},\textit{o}C6B NPs displayed strong NIR-II signal even with 1250 nm LP filter, while no signals were observed in ICG [Fig. 4(g)]. To attest the penetration depth, the NIR-II fluorescence signals of 2TT-\textit{m},\textit{o}C6B NPs and ICG under different thickness of chicken tissues were detected [Fig. 4(h)]. A high penetration depth of 8 mm was confirmed in 2TT-\textit{m},\textit{o}C6B NPs, while the fluorescence signal of ICG could hardly be detected under 4 mm chicken tissue. All these data suggested the superb NIR-II imaging quality of 2TT-\textit{m},\textit{o}C6B NPs than that of ICG.
Blood vessel imaging

**FIG. 5.** NIR-II fluorescence imaging of blood vessels in (a) hindlimb and (c) brain using 2TT-\textit{m},\textit{o}C6B NPs and ICG, respectively. (b, d) The fluorescence signal at the cross-section in hindlimb and brain (along the red-dashed line), respectively.

To prove the in vivo imaging quality of 2TT-\textit{m},\textit{o}C6B NPs, live mouse hindlimb and brain blood vessel imaging was performed through tail vein injection of agents. 2TT-\textit{m},\textit{o}C6B NPs showed an improved resolution compared with ICG, indicating that NIR-II fluorescence with a high brightness could guarantee an enhanced degree of clarity in imaging [Fig. 5(a) and (c)]. Moreover, even eight hours after administration, the blood vessels could be facilely identified (Figures 19, 20 of the supplementary materials), suggesting that 2TT-\textit{m},\textit{o}C6B NPs are promising for long-term angiography. From the fluorescence signal at the cross-section of target vasculature, 2TT-\textit{m},\textit{o}C6B NPs displayed much sharper PL spectra than that of ICG, attaining excellent clarity for in vivo imaging [Fig. 5(b) and (d)]. 2TT-\textit{m},\textit{o}C6B NPs were found to be taken up by the mononuclear phagocytic system and to accumulate in the liver and spleen (Figure 21 of the supplementary material). Altogether, benefited from the high fluorescence brightness of the NIR-II AIE dots, NIR-II fluorescence holds promise for high-resolution, real-time, deep-tissue and long-term vasculature visualization.

**Image-guided lymph node surgery**
FIG. 6. In vivo NIR-II fluorescence imaging of the lymphatic system with 2TT-m,oC6B NPs. (a) Bright field. (b-e) Fluorescent field with images captured at 10 min, 2 h, 4 h, 24 h, respectively. (f) Bright (left) and fluorescent (right) images of sentinel lymph node extracted from the mouse under the guidance of NIR-II fluorescence.

Over the last few decades, the sentinel lymph node (SLN) hypothesis has proven feasible in selected types of cancer such as breast cancer, indicating that the pathological status of the first lymph node receiving the drainage directly from a tumor is sufficient to assess cancer metastasis.54-56 SLN mapping has been extensively utilized in the intraoperative staging of many solid tumors, such as breast cancer and melanoma.57 Recently, fluorescence imaging of lymph nodes has been extensively studied.58 Owing to low autofluorescence and high photon penetration of NIR-II light, 2TT-m,oC6B NPs were considered for in vivo imaging of lymph nodes. As shown in Fig. 6(a), 50 µL of 2TT-m,oC6B NPs (1 mg/mL) was subcutaneously injected into the footpads of nude mice. After 10 min of injection, 2TT-m,oC6B NPs migrated into the lymphatic vasculature and then lighted up the SLN [Fig. 6(b)]. At two hours of post-injection, the fluorescence signal at SLN intensified with the appearance of a second lymph node [Fig. 6(c)]. Notably, the lymphatic duct between two lymph nodes could be visualized. The fluorescence signal in these two lymph nodes intensified with time and maximized at 24 h [Fig. 6(d) and (e)]. Under the guidance of
the NIR-II fluorescence, the SLNs with diameter less than 1mm were precisely removed [Fig. 6(f)], which were validated by hematoxylin and eosin (H&E) histological staining (Figure 22 of the supplementary material).

Image-guided cancer surgery

![Image](image-url)

**FIG. 7.** Tumor resection with and without 2TT-m,oC6B NPs image-guided surgery. (a) Bioluminescence and NIR-II imaging of the abdominal cavity before and after tumor resection. (b) Bioluminescence and NIR-II fluorescence signals of removed nodules of unguided and 2TT-m,oC6B NPs guided groups. (c) Histogram of nodules diameters resected from fluorescence guided and unguided groups.

Optical image-guided surgery has been proven efficient to improve surgery outcomes, which is demonstrated by some dyes such as ICG in patients. However, the short blood circulation time, limited brightness, and fast photobleaching of these dyes may result in unreliable guidance, especially for those tumors with small size (< 1mm). Alternatively, the high brightness, long blood circulation time and outstanding photostability of AIE NPs may provide a promising platform for surgical navigation.

In order to manifest the feasibility of 2TT-m,oC6B NPs to differentiate stubborn tumors, the peritoneal carcinomatosis-bearing mouse model was utilized owing to the presence of numerous tumor modules with various sizes in the peritoneal cavity. In general, it is hard to clinically identify and resect the submillimeter tumors, which may be a significant cause of cancer recurrence and metastasis. To accurately track the tumor locations, 4T1 tumor cells expressing luciferase were injected intraperitoneally into mice, which
afforded bioluminescence to tumor modules.63,64 After the establishment of a mouse model, 2TT-\textit{m,\textit{o}}C6B NPs were administrated into mice via tail vein. At 24 h post-injection of 2TT-\textit{m,\textit{o}}C6B NPs (1 mg/mL, 200 \textmu L), the mice were euthanatized and the abdomen cavity was opened for bioluminescence and NIR-II fluorescence imaging, respectively. As displayed in Fig. 7(a), the fluorescence signal overlapped well with the bioluminescence signal, owing to the enhanced permeability and retention (EPR) effect and high-brightness of the NIR-II AIEgen NPs. Moreover, the strong NIR-II fluorescence signal permitted unambiguously delineate tiny tumors from the surrounding healthy tissues.

The application of 2TT-\textit{m,\textit{o}}C6B NPs for fluorescence-guided tumor resection was then studied. A surgeon was invited to operate. As displayed in Fig. 7(a), without the guidance of the fluorescence signal, the surgeon removed lots of relatively large tumor nodules with diameters > 1mm based on his experience. Nevertheless, there were still many scattered fluorescence signals in the peritoneal cavity after the unguided surgery, indicating the remaining of tumor nodules. A second round of surgery was performed under the direction of NIR-II fluorescence signal to remove small tumor nodules. Under the high brightness of 2TT-\textit{m,\textit{o}}C6B NPs, the surgeon almost completely removed tumor nodules in fluorescence signal-guided surgery [Fig. 7(a), right]. The bioluminescence and fluorescence signals of the removed nodules overlapped thoroughly [Fig. 7(b)], indicating the accuracy of the surgery, which was also confirmed by the H&E staining image of the resected tumor nodules (Figure 23 of the supplementary material). It should be noted that the surgeon resected much smaller tumor nodules with diameter < 1 mm than that of the unguided group [Fig. 7(c)], indicating enhanced surgery accuracy by NIR-II fluorescence.

**Conclusions**

We have successfully proposed an approach of designing Janus NIR-II fluorophores by introducing electronic donors with twisted and planar structures into two flanks of central electronic acceptor motif. On the one hand, the planar architecture gives molecule excellent light-absorbing ability but suffers from fluorescence quenching effect when aggregated as a result of strong intermolecular \pi-\pi interactions. On the other hand, the twisted structure yields strong fluorescence emission in the aggregated state because of restriction of intermolecular interactions, however, a decrease in absorption is observed. Herein, as a proof-of-concept, Janus molecule 2TT-\textit{m,\textit{o}}C6B with both twisted and planar electronic donors was developed to regulate the absorption and emission properties. To our delight, the resulting 2TT-\textit{m,\textit{o}}C6B NPs inherited the merits of planar and twisted parent compounds, displaying strong absorption ($\varepsilon$ of 1.12 $\times$ 10$^4$ M$^{-1}$ 774 nm) and emission (NIR-II QY = 3.7% with an emission peak of 1050 nm) properties in the aggregated state. Thanks to the high QY and $\varepsilon$, 2TT-\textit{m,\textit{o}}C6B NPs have demonstrated outperformed in vivo imaging quality than the clinically used ICG. Notably, the 2TT-\textit{m,\textit{o}}C6B NPs helped surgeons to improve the surgical accuracy by delineating small lymph nodes or tumor nodulus. With insights from molecular design, NIR-
II biomedical imaging with improved brightness is efficient for visualizing much deeper tissues compared to visible (400-680 nm) and NIR-I (700-900 nm) imaging owing to the reduced light scattering and autofluorescence. In addition to fluorescence imaging, the strong NIR light-absorbing ability may endow 2TT-\textit{m, oC6B} NPs with excellent photothermal conversion properties, which may find applications in photoacoustic imaging, photothermal therapy (PTT), NIR-II fluorescence-guided PTT or seawater desalination. Overall, the present study provides a rational design strategy of establishing the structure-property relationship of NIR-II probes to control their emission and absorption properties.

**Supplementary materials**

See the supplementary material for more details on the molecular synthesis and characterization.

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**Data availability statement**

The data that supports the findings of this study are available within the article and its supplementary material.

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A two-in-one Janus NIR-II AIEgen with balanced absorption and emission for image-guided precision surgery

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Supplementary Note 1. Materials
All the chemicals and reagents were purchased from chemical sources, and the solvents for chemical reactions were distilled before use. Benzo[1,2-c:4,5-c’]bis([1,2,5]thiadiazole) was purchased from Derthon Optoelectronic Materials Science Technology Co LTD. TTB was purchased from AIEGEN Biotech Co., Ltd. All air and moisture sensitive reactions were carried out in flame-dried glassware under a nitrogen atmosphere.

Supplementary Note 2. Measurements
The UV-Vis-NIR absorption spectra were performed using a PerkinElmer Lambda 365 spectrophotometer. $^1$H and $^{13}$C spectra were recorded at room temperature on a Unity-400 NMR spectrometer using CDCl$_3$ as solvent and tetramethylsilane (TMS) as a reference. Mass spectra (MS) were measured with a GCT premier CAB048 mass spectrometer in MALDI-TOF mode. The photoluminescence (PL) spectra were conducted on a Horiba Fluorolog-3 spectrofluorometer. Density functional theory (DFT) calculations were carried out by the B3LYP/6G(d), Gaussian 09 package.$^1$

Supplementary Note 3. Determination of fluorescence quantum (QY) yield of the dyes.
The QY of the dyes was measured in a similar way to the previous report, using NIR-II fluorescent IR-26 dye as the reference (QY = 0.5%).$^{2,3}$ For reference calibration, IR-26 dissolved in 1,2-dichloroethane (DCE) was diluted to a DCE solution to prepare five samples with their absorbance value at 808 nm of ~0.1, ~0.08, ~0.06, ~0.04, and ~0.02, given that these highly diluted samples can minimize the second optical processes such as re-absorption and re-emission effects. Then a total of five IR-26 solutions in DCE with linearly spaced concentrations were transferred into 10-mm path fluorescence cuvette at a time. The excitation source was an 808-nm diode laser. The emission was collected in the transmission geometry with a 1000-nm long-pass filter to reject the excitation light and emission spectrum was taken in the 1000 to 1500 nm region. The same procedures were carried out for other AIEgens in H$_2$O solutions, too. Then all emission spectra of both the reference and the samples were integrated into the 1000 to 1500 nm NIR-II region. The integrated NIR-II fluorescence intensity was plotted against absorbance at the excitation wavelength of 808 nm and fitted into a linear function. Two slopes, one obtained from the reference of IR-26 in DCE and the other from the sample (AIEgens), were employed in the calculation of the QY of the sample, based on equation (1) as follows: $QY_{sample} = QY_{ref} \cdot \frac{\text{slope}_{sample}}{\text{slope}_{ref}} \cdot \left(\frac{n_{sample}}{n_{ref}}\right)^2$
where $n_{sample}$ and $n_{ref}$ are the refractive indices of H$_2$O and DCE, respectively.
**Supplementary Note 4. Fabrication of 2TT-\(m,o\)C6B NPs.**
A mixture of 2TT-\(m,o\)C6B (1 mg), DSPE-PEG\(_{2000}\) (1.5 mg) and THF (1mL) was sonicated (12 W output, XL2000, Misonix Incorporated, NY) to obtain a clear solution. The mixture was quickly injected into 9 mL of water, which was sonicated vigorously in water for 2 min. the mixture was stirred in fume food for 12 h to remove the THF. 2TT-\(m,o\)C6B NPs suspension was performed for ultrafiltration (molecule weight cutoff 100 kDa) at 3000 g for 30 min.

**Supplementary Note 5. Animals and Tumor Xenograft Model**
To establish the peritoneal carcinomatosis-bearing mouse model, a total of 800,000 luciferase-expressed 4T1 cancer cells suspended in PBS buffer were intraperitoneally injected into the Balb/c mice. Small tumor nodules were formed and scattered in the mouse peritoneal cavity after 5 days.

**Supplementary Note 6. In vitro and in vivo NIR-II fluorescence imaging**
Imaging was carried out on a home-built imaging set-up consisting of a 2D InGaAs camera (Princeton Instruments, 2D OMA-V). The excitation source was an 808 nm laser. The power density of the excitation laser at the imaging plane was 20 mW/cm\(^2\), which is significantly lower than the reported safe exposure limit of 329 mW cm\(^{-2}\) at 808 nm.

**Supplementary Note 7. In vitro NIR-II fluorescence imaging of 2TT-\(m,o\)C6B NPs and ICG**
The NIR-II fluorescence imaging system was applied to collect the NIR-II fluorescent signals of 2TT-\(m,o\)C6B NPs and ICG using different LP filters (1000, 1100, 1250 nm) under 808 nm irradiation.

**Supplementary Note 8. In vivo NIR-II Fluorescence Imaging of blood vessels.**
The NIR-II fluorescence of blood vessels in brain and hindlimb were imaged utilizing 1250 nm LP filter. After intravenous injection of 2TT-\(m,o\)C6B NPs and ICG, respectively, the in vivo NIR-II fluorescence of blood vessels in brain and hindlimb were imaged at predetermined time points (5 min, 2 h, 4 h, 6 h, 8 h and 24 h).

**Supplementary Note 9. In vivo biodistribution study.**
The biodistribution of 2TT-\(m,o\)C6B NPs and ICG in mice were studied. After injections for 24 h, the mice were sacrificed and their major tissues (heart, liver, spleen, lung, kidney and brain) were isolated and imaged.

**Supplementary Note 10. NIR-II Fluorescence Image-Guided Surgery.**
At 12 h post-injection of 2TT-\(m,o\)C6B NPs (1 mg/mL, 200 μL) into the peritoneal carcinomatosis-bearing mice via the tail vein, D-luciferin suspended in PBS buffer was intraperitoneally injected
into the peritoneal carcinomatosis-bearing mice, 5 minutes later, the mice were euthanatized and the abdomen cavity of mice was opened for bioluminescence and NIR-II fluorescence imaging. Bioluminescence imaging was performed using the Xenogen IVIS® Lumina II system, while the NIR-II fluorescence imaging was carried out using the home-built NIR-II imaging instrument. The tumor resection surgery was firstly performed by the experience of a surgeon without imaging guidance (unguided), followed by a second surgery to the same mice by the guidance of NIR-II fluorescence imaging. The excised tumor nodules were analyzed by both NIR-II fluorescence imaging and bioluminescence imaging. The tumor sizes resected from the first and second surgery were also quantified.

**Supplementary Note 11. In vivo NIR-II Fluorescence Imaging of the lymphatic system.**

Before the experiments, adult nude mice (6-8 weeks old) were anesthetized with avertin (2,2,2-Tribromoethanol, 250 mg/kg, IP). After subcutaneous injection (1 mg/mL, 50 μL) of 2TT-oC6B NPs in the footpads, the lymphatic system of the nude mice was imaged immediately at the predefined time points. At 24 h post-injection of AIEgen NPs, after carefully dissecting the superficial skin, the lymph nodes were dissected out by the guidance of NIR-II fluorescence imaging and imaged under bright field and 808 nm laser excitation conditions, respectively.

**Supplementary Note 12. Histological Study.**

The excised tumor nodules and lymph nodes by the surgeon from mice were performed for histological analysis. Briefly, the tissues were fixed in 4% paraformaldehyde, processed routinely into paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin and eosin (H&E). The slices were imaged by a digital microscope (Leica QWin).

**Supplementary Note 13. Animal ethics.**

All animal experiments were performed in compliance with the guidelines set by Tianjin Committee of Use and Care of Laboratory Animals and the overall project protocols were approved by the Animal Ethics Committee of Nankai University. All the mice were obtained from Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China).
Supplementary Scheme 1. Synthetic route to 2TT-mC6B.

**Synthetic route of 2.**

Add nBuLi (2.3 mL, 5.8 mmol, 2.4 M in hexane) dropwise to a solution of 3-hexylthiophene (0.83 g, 5.2 mmol) in THF (30 mL) at -78 °C. Stirring the reaction mixture 1 h at -78 °C. Then tributyltin chloride (1.8 g, 5.8 mmol) was added into the reaction at one portion. After stirring the mixture for 12 h at room temperature, KF solution was added to quench the reaction. The mixture was extracted with hexane three times, the combined organic phase was dried with Na₂SO₄. After removing the solvent, the product was used directly without further purification.

**Synthetic route of 3.**

Under N₂ atmosphere, 1 (87 mg, 0.25 mmol), 2 (343 mg, 0.75 mmol), Pd(PPh₃)₄ (22 mg, 0.025 mmol), and 20 mL toluene were added to a 100 mL predried two-necked flask. The mixture was refluxed for 24 h. After cooling down to room temperature, the solvent was removed by rotary evaporation. The crude product was purified by silica gel column to obtain the target molecule yield, 40%. ¹H NMR (400 MHz, CDCl₃) δ 8.85 (2H, s), 7.32 (2H, s), 2.79 (4H, d, J = 8Hz), 1.77 (4H, m), 1.4-1.2 (12H, m), 0.89 (6H, m).
Supplementary Figure 1. $^1$H NMR spectrum of 3.

**Synthetic route of 4.**

Compound 3 (0.17 g, 0.33 mmol) was dissolved in a mixture of 10 mL CHCl$_3$ and 10 mL acetic acid under argon atmosphere, NBS (117 mg, 6.6 mmol) was added slowly over the course of 30 mins in a mixture of 5 mL CHCl$_3$ and 5 mL acetic acid at room temperature under the exclusion of light. The mixture was stirred overnight and was then dried by condensed air. The crude product was purified by silica gel column to obtain the product (yield: 90%). $^1$H NMR (400 MHz, CDCl$_3$), δ (ppm): 8.76 (2H, s), 2.73 (4H, d, J= 8Hz), 1.76 (4H, m), 1.4-1.2 (12H, m), 0.89 (6H, m).
Supplementary Figure 2. $^1$H NMR spectrum of 4.

**Synthetic route of 5.**

Add nBuLi (2.3 mL, 5.8 mmol, 2.4 M in hexane) dropwise to a solution of 4-bromotriphenylamine (1.68 g, 5.2 mmol) in THF (30 mL) at -78 °C. Stirring the reaction mixture 1 h at -78 °C. Then tributyltin chloride (1.8 g, 5.8 mmol) was added into the reaction at one portion. After stirring the mixture for 12 h at room temperature, KF solution was added to quench the reaction. The mixture was extracted with hexane three times, the combined organic phase was dried with Na$_2$SO$_4$. After removing the solvent, the product was used directly without further purification.

**Synthetic route of 2TT-mC6B.**

Under N$_2$ atmosphere, 4 (170 mg, 0.25 mmol), 5 (400 mg, 0.75 mmol), Pd(PPh$_3$)$_4$ (22 mg, 0.025 mmol), and 20 mL toluene were added to a 100 mL predried two-necked flask. The mixture was refluxed for 24 h. After cooling down to room temperature, the solvent was removed by rotary evaporation. The crude product was purified by silica gel column to obtain the target molecule yield, 30%). $^1$H NMR (400 MHz, CDCl$_3$), δ (ppm): 8.93 (2H, s), 7.47 (2H, m), 7.32-7.27 (10H, m), 7.18-7.12 (10H, m), 7.09-7.0 (4H, m), 2.87 (4H, m), 1.83 (4H, m), 1.26 (12H, m), 0.89 (6H, m). $^{13}$CNMR (100 MHz, CDCl$_3$), δ (ppm): 150.6, 146.8, 146.7, 143.4, 138.9, 135.0, 132.4, 129.1, 128.7, 128.0, 125.5, 124.1, 123.9, 123.5, 122.6, 122.0, 112.3, 30.4, 29.0, 28.7, 22.1, 20.6, 13.5, 13.1. MS: m/z: [M]+ calcd for C$_{62}$H$_{56}$N$_6$S$_4$: 1012.3449, found: 1012.3419.
Supplementary Figure 3. $^1$H NMR spectrum of 2TT-$m$C6B.

Supplementary Figure 4. $^{13}$C NMR spectrum of 2TT-$m$C6B.
Supplementary Figure 5. MALDI-TOF-MS spectrum of 2TT-\textit{m}C6B.

Supplementary Scheme 2. Synthetic route to 2TT-\textit{o}C6B.
Synthetic route of 7.

3- Hexylthiophene, 6 (2.6 g, 0.016 mol) was dissolved in a mixture of chloroform and acetic acid (1:1, 50 mL), and N-bromosuccinimide (2.6 g, 0.016 mol) was added slowly at room temperature. The compound was extracted with hexane (3×50 mL), the combined organic layer was washed with water (3×100 mL), dried over anhydrous Na2SO4 and concentrated to yield a yellow oil which was purified by column chromatography on silica gel using hexane as the eluent to obtain a clear oil (yield: 96%). 1H NMR (400 MHz, CDCl3), δ (ppm) = 7.19 (1H, d, J = 2Hz), 6.80 (1H, d, J = 2Hz), 2.56 (2H, m), 1.58 (2H, m), 1.35 (6H, m).

Supplementary Figure 6. 1H NMR spectrum of 7.

Synthetic route of 8.

Add nBuLi (2.3 mL, 5.8 mmol, 2.4 M in hexane) dropwise to a solution of 7 (1.28 g, 5.2 mmol) in THF (30 mL) at -78 °C. Stirring the reaction mixture 1 h at -78 °C. Then tributyltin chloride (1.8 g, 5.8 mmol) was added into the reaction at one portion. After stirring the mixture for 12 h at room temperature, KF solution was added to quench the reaction. The mixture was extracted with hexane three times, the combined organic phase was dried with Na2SO4. After removing the solvent, the product was used directly without further purification.
**Synthetic route of 9.**

Under N$_2$ atmosphere, 1 (87 mg, 0.25 mmol), 8 (343 mg, 0.75 mmol), Pd(PPh$_3$)$_4$ (22 mg, 0.025 mmol), and 20 mL toluene were added to a 100 mL predried two-necked flask. The mixture was refluxed for 24 h. After cooling down to room temperature, the solvent was removed by rotary evaporation. The crude product was purified by silica gel column to obtain the target molecule yield, 40%). $^1$H NMR (400 MHz, CDCl$_3$) δ 7.65 (2H, s), 7.25 (2H, s), 2.6 (4H, d, J = 8Hz), 1.77 (4H, m), 1.4-1.2 (12H, m), 0.73 (6H, m).

**Supplementary Figure 7.** $^1$H NMR spectrum of 9.

**Synthetic route of 10.**

Compound 9 (0.17 g, 0.33 mmol) was dissolved in a mixture of 10 mL CHCl$_3$ and 10 mL acetic acid under argon atmosphere, NBS (117 mg, 6.6 mmol) was added slowly over the course of 30 mins in a mixture of 5 mL CHCl$_3$ and 5 mL acetic acid at room temperature under the exclusion of light. The mixture was stirred overnight and was then dried by condensed air. The crude product was purified by silica gel column to obtain the product (yield: 90%). $^1$H NMR (400 MHz, CDCl$_3$), δ (ppm): 7.2 (2H, s), 2.51 (4H, d, J= 8Hz), 1.76 (4H, m), 1.4-1.2 (12H, m), 0.89 (6H, m).
Supplementary Figure 8. $^1$H NMR spectrum of 10.

**Synthetic route of 2TT-oC6B.**

Under N$_2$ atmosphere, 10 (170 mg, 0.25 mmol), 5 (400 mg, 0.75 mmol), Pd(PPh$_3$)$_4$ (22 mg, 0.025 mmol), and 20 mL toluene were added to a 100 mL predried two-necked flask. The mixture was refluxed for 24 h. After cooling down to room temperature, the solvent was removed by rotary evaporation. The crude product was purified by silica gel column to obtain the target molecule yield, 32%). $^1$H NMR (400 MHz, CDCl$_3$), δ (ppm) = 7.59-7.56 (4H, m), 7.37 (2H, s), 7.31-7.26 (8H, m), 7.16-7.12 (8H, m), 7.11-7.03 (8H, m), 2.61-2.57 (4H, t, J = 8Hz), 1.63, (4H, m), 1.15-1.10 (12H, m), 0.73 (6H, m). $^{13}$C NMR (100 MHz, CDCl$_3$), δ (ppm): 152.6, 146.9, 146.8, 146.3, 145.0, 128.7, 127.5, 126.1, 124.0, 122.7, 122.5, 115.4, 99.3, 30.8, 29.8, 29.6, 28.4, 21.8, 13.3. MS: m/z: [M]+ calcd for C$_{62}$H$_{56}$N$_{6}$S$_{4}$: 1012.3449, found: 1012.3.
Supplementary Figure 9. $^1$H NMR spectrum of 2TT-oC6B.

Supplementary Figure 10. $^{13}$C NMR spectrum of 2TT-oC6B.
Supplementary Figure 11. MALDI-TOF-MS spectrum of 2TT-oC6B.

Supplementary Scheme 3. Synthetic route to 2TT-m,oC6B.
**Synthetic route of 11.**

Under N\(_2\) atmosphere, 1 (87 mg, 0.25 mmol), 2 (170 mg, 0.37 mmol), 8 (170 mg, 0.37 mmol), Pd(PPh\(_3\))\(_4\) (22 mg, 0.025 mmol), and 20 mL toluene were added to a 100 mL predried two-necked flask. The mixture was refluxed for 24 h. After cooling down to room temperature, the solvent was removed by rotary evaporation. The crude product was purified by silica gel column to obtain the target molecule yield, 20%. \(^1\)H NMR (400 MHz, CDCl\(_3\)), \(\delta\) (ppm) = 7.66-7.56 (2H, d, J = 2Hz), 7.25 (2H, m), 2.58-2.54 (4H, m), 1.52 (4H, m), 1.26-1.10 (12H, m), 0.73 (6H, m).

**Supplementary Figure 12. \(^1\)H NMR spectrum of 11.**

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**Synthetic route of 12.**

Compound 10 (0.17 g, 0.33 mmol) was dissolved in a mixture of 10 mL CHCl\(_3\) and 10 mL acetic acid under argon atmosphere, NBS (117 mg, 6.6 mmol) was added slowly over the course of 30 mins in a mixture of 5 mL CHCl\(_3\) and 5 mL acetic acid at room temperature under the exclusion of light. The mixture was stirred overnight and was then dried by condensed air. The crude product was purified by silica gel column to obtain the product (yield: 93%). \(^1\)H NMR (400 MHz, CDCl\(_3\)), \(\delta\) (ppm): 8.78 (1H, s), 7.17 (1H, s), 2.73 (2H, d, J = 8 Hz), 2.46 (2H, d, J = 8Hz), 1.75 (2H, m), 1.4-1.1 (14H, m), 0.91 (3H, m), 0.73 (3H, m).
Supplementary Figure 13. $^1$H NMR spectrum of 12.

![NMR Spectrum of 12](image)

**Synthetic route of 2TT-m,oC6B.**

Under N$_2$ atmosphere, 12 (170 mg, 0.25 mmol), 5 (400 mg, 0.75 mmol), Pd(PPh$_3$)$_4$ (22 mg, 0.025 mmol), and 20 mL toluene were added to a 100 mL predried two-necked flask. The mixture was refluxed for 24 h. After cooling down to room temperature, the solvent was removed by rotary evaporation. The crude product was purified by silica gel column to obtain the target molecule yield, 29%. $^1$H NMR (400 MHz, CDCl$_3$), $\delta$ (ppm) = 8.95 (1H, s), 7.61 (2H, d, J = 8Hz), 7.54 (2H, d, J = 8Hz), 7.37-7.26 (9H, m), 7.23-7.06 (16H, m), 2.88 (2H, d, J = 8Hz), 2.57 (2H, d, J = 8Hz), 1.75 (2H, m), 1.4-1.1 (14H, m), 0.91 (3H, m), 0.73 (3H, m). $^{13}$C NMR (100 MHz, CDCl$_3$), $\delta$ (ppm): 153.9, 150.4, 147.6, 147.4, 146.3, 145.2, 145.1, 139.7, 136.5, 135.1, 129.8, 129.4, 129.3, 128.6, 128.4, 128.0, 126.7, 124.8, 124.6, 123.4, 123.3, 123.1, 122.9, 115.4, 113.0, 31.7, 31.6, 31.5, 31.0, 30.5, 29.3, 29.1, 29.0, 22.7, 22.5, 14.1, 14.0. MS: m/z: [M]+ calcd for C$_{62}$H$_{56}$N$_6$S$_4$: 1012.3449, found: 1012.3325.
Supplementary Figure 14. $^1$H NMR spectrum of 2TT-$m,o$C6B.

Supplementary Figure 15. $^{13}$C NMR spectrum of 2TT-$m,o$C6B.
Supplementary Figure 16. MALDI-TOF-MS spectrum of 2TT-\textit{m, o}C6B.

Supplementary Figure 17. PL spectra of (a) 2TT-\textit{m}C6B and (b) 2TT-\textit{o}C6B in THF/H\textsubscript{2}O mixture with different \(f_\text{w}\).
Supplementary Figure 18. Dynamic light scattering profile of 2TT-\textit{m,}{\textit{o}}C6B NPs.
Supplementary Figure 19. NIR-II fluorescence imaging of blood vessels in hindlimb at different time via tail vein injection of 2TT-\textit{m,\textit{o}}C6B NPs (1 mM, 200 µL) and ICG (1 mM, 200 µL), respectively.
Supplementary Figure 20. NIR-II fluorescence imaging of blood vessels in brain at different time via tail vein injection of 2TT-m,0C6B NPs (1 mM, 200 µL) and ICG (1 mM, 200 µL), respectively.

Supplementary Figure 21. Biodistribution profile of fluorescent 2TT-m,0C6B NPs and ICG in ex vivo tissue imaging.
Supplementary Figure 22. H&E staining image of the resected lymph node.

Supplementary Figure 23. H&E staining image of the resected tumor nodule.
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