**Targeted Multicolor *In Vivo* Imaging Over 1000 nm Enabled by Nonamethine Cyanines**

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**Abstract**

Recent progress has shown that using wavelengths between 1000 and 2000 nm, referred to as the Shortwave-Infrared (SWIR) or Near-Infrared (NIR)-II range, can enable high-resolution in vivo imaging at depths not possible with conventional optical wavelengths. However, few bioconjugatable probes of the type that have proven invaluable for multiplexed imaging in the visible and near-infrared range are available for imaging these wavelengths. Using rational design, we have generated persulfonated indocyanine dyes with absorbance maxima at 872 and 1072 nm through catechol- and aryl-ring fusion, respectively, onto the nonamethine scaffold. Multiplexed two- and three-color *in vivo* imaging using monoclonal antibody (mAb) and dextran conjugates in several tumor models illustrate the benefits of concurrent labeling of the tumor and healthy surrounding tissue and lymphatics. These efforts are enabled by complementary advances in a custom built NIR/SWIR imaging setup and software package for multicolor real-time imaging.

**Introduction**

*In vivo* fluorescence imaging has the potential to probe biological processes in their native environments.1 While multiplexed imaging is routine in microscopy, such experiments are challenging in live tissue due to the competitive autofluorescence and scattering encountered with visible (< 700 nm) and even near-infrared (NIR) wavelengths (700-1000 nm).2,3 Intravital microscopy using two-photon excitation enables multicolor imaging of tissue sections in living animals.4 However, these methods require exteriorization of the organ of interest or implantation of an imaging window and, consequently, are not amenable to body-wide imaging. Another alternative, NIR imaging, relies on the narrow optical imaging window compatible with tissue and standard silicon-based detectors. Recent progress has demonstrated the benefits of imaging using longer wavelengths (1000 to 2000 nm), referred to as the Shortwave-Infrared (SWIR) or NIR-II range. These methods enable high-resolution imaging in bulk tissue, with dramatic improvements in both resolution and depth relative to imaging in the NIR region.5 As InGaAs detector-based imaging systems have become more readily available, a critical bottleneck in this field is access to biologically compatible fluorescent probes that operate in this range.6,7 Foundational studies in this field used SWIR emissive carbon nanotubes,8,9 quantum dots,10,11 rare-earth−doped nanocomposites,12 gold nanomaterials13, and indocyanine green (ICG).14,15 Water soluble, targetable small molecule fluorophores are readily amenable to clinical translation and remain the most common exogenous probes for biological research. Bioconjugatable probes with high absorption coefficients, well-separated absorbance maxima, and efficient emission in the SWIR region – properties critical for targeted *in vivo* multiplexing experiments – are still needed.16-19

 Indocyanine dyes are broadly used in multiplexed fluorescence-based experiments. In the visible range, trimethine indocyanines (e.g. Cy3) and pentamethine indocyanines (e.g. Cy5, AF-647), find extensive use in applications that range from routine quantification to advanced microscopy.20-23 Derivatives of heptamethine-indocyanines (e.g. Cy7, IR-800CW and ICG) are the most broadly applied molecules for *in vivo* NIR imaging,24 including for clinical use.25,26 Heptamethine cyanines were also recently found to exhibit significant emission beyond 1000 nm, which has been validated in numerous settings, including clinically with ICG.14,15,27-30 There have also been several recent reports on novel cyanine-type dyes bearing different heterocycles with absorbance and emission maxima in the SWIR range, although these have been limited to untargeted probes to date.31-34 Furthermore, while a handful of hydrophobic, unmodified nonamethine cyanine dyes have been reported, no variants had been examined for direct conjugation for biological applications.35 Our prior studies tuning the heptamethine scaffold convinced us that suitably substituted nonamethine indocyanines could be broadly useful SWIR dyes for targeted imaging.24,35,36

 This report details our efforts to extend the indocyanine scaffold to longer wavelengths for fast multiplexed *in vivo* imaging applications. Enabled by rational computational design, we report two novel classes of targeted nonamethine indocyanine dyes with significant emission in the SWIR range (>1000 nm) and red-shifted absorbance maxima compared to the clinically employed dyes ICG and IR-800CW **(Fig.** **1a**). The first are catechol derivatives that exhibit absorbance maxima around 870 nm and the second involve modification of the nonamethine cyanine scaffold with a fused aryl ring, which extends the absorbance maximum beyond 1000 nm. These highly water-soluble dyes are readily targetable and exhibit SWIR emission and photochemical and hydrolytic stability on par with or exceeding that of existing heptamethine cyanines. Targeted tumor imaging was successfully performed using FNIR-872-mAb and FNIR-1072-mAb, revealing tumor to background ratios (TBR) comparable to the clinically used IR-800CW-mAb. We demonstrate the application of these mAb conjugates in combination with dextran bioconjugates and ICG to delineate the tumor mass, surrounding lymphatic vessels and tumor vasculature in multiple mouse tumor models. We also developed a dedicated imaging instrument and software interface to enable real-time multiplexed imaging with three fluorescent channels and one reflection channel, the different fluorescence signals were clearly separated with negligible crosstalk. Collectively, these advances in dye chemistry and instrumentation enable simultaneous visualization of tumor tissue, draining lymphatic structures, and sentinel lymph nodes during a very dynamic procedure such as fluorescence-guided surgery, requiring video-rate acquisition.

**Results**

**Computational design, synthesis and characterization.** To aid in the design of substituted nonamethine cyanines, we first carried out a computational analysis to probe the effect of substituents on a model nonamethine scaffold. These studies suggested that the combination of catechol fusion and dehydrodecaline substitution leads to a planar polymethine chromophore with conventional cyanine-like HOMO and LUMO orbitals, and a predicted absorbance maximum of 933 nm. More dramatically, aryl ring fusion at the C4’/C6’ positions, provided a predicted absorbance maximum over 1000 nm due to stabilizing increased LUMO density on the appended aryl group (**Fig. 1b**. **Supplementary Note 1**).

 Persulfonated variants of these compounds were accessed in a concise sequence. The tetrasulfonated dichloro derivative **1**, which we anticipated would be a useful synthetic intermediate in route to the calculated structures, was prepared over two steps (**Supplementary Note 2**). Compound **1** was then functionalized by reaction with 3,4-dihydroxybenzoic acid **2** under aqueous conditions (PBS) to afford FNIR-866 in 64% yield or by Suzuki cross-coupling with bis-boronate **3** to obtain FNIR-1072 in 35% yield (**Fig. 1c**).

The spectral and photophysical properties of FNIR-866 and FNIR-1072 were examined in detail (**Fig. 1 d,e, Supplementary Figs. 1 and 2**). The emission of probes in this wavelength range is reduced by competitive deactivation of the excited state by solvent – a trend which we also observe.37 For example, FNIR-866 has a brightness value ( × F) of 7,300 (MeOH), which is ~45% of ICG (16,000).38 We also examined the photostability of these compounds when irradiated with LEDs tuned to their absorbance maxima. We found that while IR-800CW and FNIR-866 exhibit similar photostability, FNIR-1072 undergoes only minimal photobleaching (<5%) under these conditions (**Extended Data Fig. 1**). The latter observation is consistent with prior studies that suggest cyanine photobleaching in an aqueous environment is largely mediated through a self-sensitized 1O2 mechanism, which is likely less accessible to the low-energy triplet states of FNIR-1072.39 This hypothesis is consistent with a reduction in singlet oxygen quantum yield () for FNIR-1072 relative to FNIR-866 and structurally related heptamethine cyanines (**Fig. 1e**).40 We also examined their chemical stability in serum by monitoring the absorption at the respective absorption maxima over time, and found these dyes to be quite stable, with no distinguishable difference relative to IR-800CW (**Extended Data Fig. 2**).

To facilitate multiplexed imaging of the nonamethine cyanines alongside heptamethine cyanines, we developed a custom-built imaging system (**Supplementary Note 3**)33 that incorporates three different NIR laser excitation sources, one for each of the three dyes (λex = 785 nm for heptamethine cyanine dyes, λex = 892 nm for FNIR-866 and λexcitation = 968 or 1064 nm for benzo-fused Cy9 dye FNIR-1072), as well as a 1300 nm LED for reflectance imaging. We use long-pass emission filters (as specified) which detect the emission tail of these dyes. When excited with their respective laser lines at equal power densities (90 mW/cm2) and imaged with a 1150 nm long-pass emission filter our nonamethine cyanine dyes had SWIR tail emission that was comparable to, but slightly greater than commercial heptamethine cyanine dyes ICG, IR-800CW and our optimized heptamethine cyanine mAb label FNIR-Tag (**Fig.** **2a**, **Extended Data Fig. 3**).41 Using optimal excitation wavelengths for each dye, we investigated the overlapping emission in each channel using dye solutions of equal concentration and found that there was considerable crosstalk in the 785 and 890 nm channels (**Fig. 2b,** left). However, efficient spectral separation can be achieved by imaging lower concentrations of the longer-wavelength dyes with progressively longer camera exposure times or by varying excitation power (**Fig. 2b**, middle, right). Additionally, these dyes can be spectrally separated with a conventional multiplexing approach based on excitation and emission wavelength by employing two different sensors with quantum efficiency spanning the NIR and SWIR (**Supplementary Fig. 3**).

Motivated by the potential for multiplexing of these two new bioconjugatable dyes alongside extensively used heptamethine cyanines (ICG and IR-800CW), we prepared the corresponding monoclonal antibody (mAb) conjugates of FNIR-866 and FNIR-1072 with the anti-EGFR mAb Panitumumab (**Supplementary Fig. 4**). In our initial labeling experiments, we found that the first generation FNIR-866 exhibited poor labeling efficiency (DOL 0.6 using 10 eq. of NHS ester) and evidence of H-aggregate formation during the conjugation.41,42 We then prepared the dye FNIR-872 through addition of a linker bearing an additional sulfonate group to improve the labeling and properties of the corresponding antibody fluorophore conjugate (**Supplementary Fig. 5)**.

**Targeted imaging with nonamethine cyanine-mAb conjugates.** We chose to benchmark FNIR-872-Pan and FNIR-1072-Pan to the clinically explored IR-800CW-panitumumab (IR-800CW-Pan).43 Each conjugate (100 μg dose, DOL 1.7 for IR-800CW, DOL 1.4 for FNIR-872, and DOL 1.0 for FNIR-1072) was injected by tail vein into athymic nude mice (n = 3 or 4) bearing MDA-MB-468 (EGFR+) xenograft tumors implanted subcutaneously (s.c.) in the right flank. All three conjugates exhibited similar tumor-targeting ability, tumor to background ratios (TBR) (**Fig. 2d,e**), and biodistribution (**Supplementary Fig. 6**), albeit with somewhat higher liver and background signals for FNIR-872 and FNIR-1072 that eventually cleared after 7 days post-injection. Additionally, the uptake of the IR-800CW-Pan and FNIR-872-Pan conjugates in the tumor tissue was confirmed by microscopy using a standard epifluorescence microscope equipped with NIR lasers (**Extended Data Figs. 4-6**). Highlighting the significant performance of these probes, we also found that FNIR-872-Pan could be used in awake mouse imaging (**Supplementary Video 1**). These results suggest that conjugates of FNIR-872 and FNIR-1072 can be used in a similar fashion to existing heptamethine cyanine dyes for *in vivo* imaging experiments in the SWIR.

SWIR imaging has been used to study blood dynamics and clearance pathways through the use of nanomaterials and small-molecule SWIR emitters formulated in polymeric materials.6,7,44,45 Dextran conjugates are also broadly used in this context, and benefit from well-defined clearance pathways.46 Therefore, we prepared SWIR-emissive blood pool agents in three colors by conjugation of FNIR-872 and FNIR-1072, in addition to our optimized heptamethine cyanine dye, FNIR-Tag,41 to the biocompatible branched polysaccharide amino-dextran (**Supplementary Note 2** and **Supplementary Fig.** **7**). In an initial two-color experiment, we injected the efficiently renally clearable FNIR-Tag-Dex (10 kDa) in a mouse bearing an MDA-MB-468 xenograft that was administered FNIR-872-Pan two days prior, enabling the simultaneous visualization of the kidneys and tumor both *in vivo* and *ex vivo* with minimal crosstalk(**Extended Data Fig. 7**).

**Targeted multicolor imaging in the SWIR.** The preferential uptake and retention of ICG has been validated in a number of solid tumor types. This approach has extensive clinical validation in the context of liver cancer, however a challenge complicating its use is the ability to distinguish tumor signal from background hepatobiliary clearance of ICG.26,47-49 We hypothesized that the combination of multicolor imaging with SWIR detection could aid in the delineation of a deep-seated liver tumor labeled with ICG from surrounding healthy liver tissue in a noninvasive manner. To test this, ICG was injected by tail vein in an athymic nude mouse bearing a Hep3B (human hepatocellular carcinoma) orthotopic tumor surgically implanted in the left liver lobe as well as a control mouse.50 Using single color NIR or SWIR imaging alone, we could not readily differentiate the tumor from the surrounding liver tissue (**Extended Data Fig. 8**). By contrast, using two color SWIR imaging we could readily identify a tumor within the liver *in vivo*, which was readily confirmed by necroscopy and *ex vivo* imaging (**Fig. 3a,b,c**). We note that in this experiment, significant emission from the FNIR-872 dextran conjugate was observed in the ICG channel, which could easily be resolved in the corresponding merged image or through spectral unmixing (**Supplementary Fig. 8**).

In addition to tumor-mass targeting, ICG has found widespread use in both fundamental and clinical contexts to image lymphatic function or to visualize the sentinel lymph node (e.g., in breast cancer patients).51 With NIR detection, the simultaneous non-invasive visualization of tumor lymphatics and tumor mass using targeted small molecule organic dyes is not possible due to the lack of targeted approach in a different absorption/emission range than ICG. We injected ICG intradermally in the tail of an orthotopic MDA-MD-468 human breast tumor bearing mouse to enable visualization of the lymphatic vessels near an *in vivo* labeled tumor using FNIR-872-Pan **(Fig. 3d,e,f** **Supplementary Video 2**). Notably, there is very little crosstalk between ICG and FNIR-872-Pan in this experiment or in a similar experiment using FNIR-1072-Pan as the tumor-targeting agent (**Extended Data Fig. 9, Extended Data Fig. 10)**. Additionally, FNIR-1072-Dex could be used in combination with ICG and FNIR-872-Pan to highlight the vasculature in the tumor surroundings (**Supplementary Video 3**) and enabled longitudinal assessment of vascular permeability and lymphatic pumping in addition to tumor visualization. Taken together, these experiments illustrate the utility of FNIR-872 and FNIR-1072 alongside ICG in high-speed multicolor *in vivo* imaging experiments.

To enable the user to visualize anatomical references in addition to fluorescence, we incorporated an LED-based reflectance channel (1300 nm), which was interleaved with the fluorescence imaging channels. We also implemented a user-friendly interface which allows all four channels to be visualized in real-time **Supplementary Note 3**). This approach also allowed us also to perform precise dissection of the tumor and lymph node guided by real-time multicolor guidance using FNIR-872 or FNIR-1072 interchangeably as the vasculature or tumor targeting agent (**Fig. 3g**, **Supplementary Videos 4-7**).

**Discussion**

Here we report the rational design, synthesis and evaluation of novel long-wavelength emitting fluorophores that result from the fusion of catechol and aryl rings onto a substituted nonamethine scaffold. These studies illustrate that rationally designed modifications to the core cyanine chromophore unit can dramatically alter optical properties, and when combined with suitable peripheral substituents maintain high chemical stability and excellent water solubility. Amine-reactive NHS esters enable the preparation of highly emissive mAb and dextran bioconjugates, which are used for a variety of *in vivo* experiments. We demonstrated that these compounds can be used alongside ICG and other clinically relevant heptamethine cyanine dyes in the first two- and three-color imaging experiments in the SWIR range exclusively using only targeted small molecule organic dyes. 17,52

Importantly, our imaging approach is not sensitive to visible light and allows for fluorescence-guided surgery with room lights on, provided that the lighting is based on visible LED or other non-SWIR-emitting technology. This simplifies current workflows, which may require dimming the room and surgical lighting, or synchronizing those lights to shutters controlled by the fluorescence imaging system. In addition to applications in cancer research, we anticipate this multicolor imaging approach could be useful for a variety of clinical applications. For example, when, first, a tumor mass needs to be resected, second, the draining lymphatics need to be followed for identification of the sentinel lymph node and, lastly, an anastomosis needs to be checked for viability by detecting its perfusion through a blood pool agent.

Existing persulfonated indocyanines (“Cy-dyes”) are fluorophores of choice for multiplexed microscopy experiments. These studies extend the applications of these molecules to multicolor *in vivo* imaging and potential future clinical translation. Here we have demonstrated the potential to simultaneously label the tumor mass in a mouse model, as well as associated tumor vasculature and lymphatic vessels. These molecules in combination with the multi-channel real time visualization tools we have developed will have applications in diverse preclinical and clinical contexts where multiplexed information is needed. Critically, the probes reported here are among the first examples of bioconjugatable fluorophores with red-shifted excitation wavelengths relative to heptamethine cyanine dyes, and enable a range of multiplexed biological studies not possible before in a body-wide live animal setting. Additional efforts to optimize the physical properties of these highly anionic scaffolds might improve their *in vivo* properties. We also envision that dynamic, stimuli responsive probes can be accessed through derivatization of these scaffolds.53

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**Author Contributions**

V.G.B., M.J.S. and O.T.B designed the project. M.J.S. and O.T.B. jointly advised the project. M.J.S. and J.I. designed and carried out the computational chemistry. V.G.B and M.P.L performed organic synthesis and characterization. B.R. and U.R.-G. performed photophysical measurements and analyzed the data. T.S.B, J.G.P.L, M.S. and M.P.L. built the optical imaging setup. M.P.L and N.L.P performed phantom imaging experiments. M.N.N and M.S. performed cell culture. M.S., C.S., C.M.R., L.R. and S.D. established animal tumor models and were responsible for animal handling and care. V.G.B., M.P.L and M.S. performed bioconjugation chemistry and characterization. M.P.L, M.S., J.D.K., N.L.P, and J.G.P.L. designed and carried out animal imaging experiments. P.T.T developed the imaging software. M.P.L and M.S. processed and analyzed the imaging data. M.P.L. and M.J.S. wrote the manuscript. All authors have reviewed and approved the final version of the manuscript. Requests for materials and other correspondence should be addressed to M.J.S and O.T.B.

**Competing Interests**

The following authors declare competing interests: V.G.B. M.J.S., J.G.P.L., M.S. and O.T.B. have filed patent applications whose value may be affected by this publication. The remaining authors declare no competing interests.

Editor’s summary

Rational design was used to develop a suite of red-shifted, bioconjugatable heptamethine cyanine dyes for multiplexed in vivo imaging in the SWIR/NIR-II region.

**Figure Captions**

**Figure 1. Synthesis and characterization of nonamethine cyanines** **a,** Prior indocyanine dyes and novel nonamethine cyanine dyes reported here. **b,** HOMO/LUMO plots for truncated FNIR-866 (left) and FNIR-1072 (right). **c,** Synthesis of FNIR-866 and FNIR-1072 by diversification of dichloro-nonamethine cyanine **1**. **d,** Normalized absorbance (solid line) and emission (dashed line) spectra (InGaAs detection) of FNIR-866 (cyan) and FNIR-1072 (magenta) in 10% FBS/PBS. **e,** Keyspectroscopic properties for FNIR-866 and FNIR-1072 obtained in 10% FBS/PBS. Singlet oxygen quantum yields (ΦΔ) were obtained in methanol.

**Figure 2. Probe comparison, multiplexing strategy and single color mAb imaging a**, Imaging solutions of indocyanine dyes (5 μM in 10% FBS/PBS) using 1150 nm long pass filters. Green, cyan and purple bars represent indocyanine derivatives that are excited with 785 nm, 890 nm or 1064 nm lasers, respectively. Image scale is 0-3000 count/ms. **b**, Left, SWIR emission of ICG, FNIR-866, and FNIR-1072 at 5 μM concentration in 10% FBS/PBS with matched power density (90 mW/cm2) and matched exposure time (10 ms), .Middle, SWIR emission of ICG (5 μM), FNIR-866 (500 nM) and FNIR-1072 (50 nM) in 10% FBS/PBS with matched power density (90 mW/cm2) and varied camera exposure time (10/30/300 ms, respectively). Right, SWIR emission of ICG (5 μM), FNIR-866 (500 nM), and FNIR-1072 (50 nM) with varied power density (10/25/250 mW/cm2, respectively) and matched camera exposure time (50 ms). Image scales are 0-15000, 500-13000, and 200-5000 counts in all three channels for the left, middle and right images, respectively. **c,** *In vivo* imaging comparison of 100 g IR-800CW-Pan (DOL 1.7), 100 g FNIR-872-Pan (DOL 1.4) and 100 g FNIR-1072-Pan (DOL 1.0) injected into the tail vein of athymic nude mice bearing MDA-MB-468 xenograft tumors implanted s.c. in their right flank at 48 h postinjection. See Supplementary Note 4 for detailed acquisition settings. **d,** Quantification of tumor to background ratio (TBR) (n = 5 animals for IR-800CW-Pan and FNIR-872-Pan, n = 3 for FNIR-1072-Pan) of all three mAb conjugates at 48 h post-injection. Error bars were derived from the standard deviation, differences are not statistically significant.

**Figure 3.** **Applications of *in vivo* multicolor optical imaging.** **a,** Dosing scheme for two-color imaging experiment of an athymic nude mouse bearing a Hep3B orthotopic liver tumor using ICG (2 mg/kg) and FNIR-872-Dex (1 nmol effective dye dose) **b**, Representative images in each channel of the Hep3B orthotopic tumor both *in vivo* and *ex vivo*. Color scales in the 785/890 channels are 0-100/0-150 and 55-205/0-250 counts/ms for the *in vivo* and *ex vivo* images, respectively. **c**, Plot profiles of each channel drawn diagonally across the length of the excised liver. **d,e** Longitudinal 3- and 4- channel multicolor imaging of tumor signal, and lymphatic pumping and tumor vascular permeability in an athymic nude mouse bearing an MDA-MB-468 orthotopic tumor implanted in the mammary fat pad using ICG (100 nmol), FNIR-872-Pan (142 μg, 1 nmol effective dye dose, DOL 1.0) and FNIR-1072-Dextran (10 kDa, 100 μg, 2 nmol effective dye dose) at the indicated time-points. Emission was collected with 1050 nm long pass filters. FNIR-1072-Dextran (10 kDa) was injected intravenously into the mouse and images in all three channels were recorded 3 minutes post-injection. Color scales in channels 1-4 are 0-15000, 0-15000,1000-10000 and 4000-40000 counts. **f**, Overlaid plot profiles of each channel from the mouse in **Fig. 3d,e** drawn diagonally across the length of the tumor (yellow line) pre- and post-injection of FNIR-1072-Dex, illustrating minimal crosstalk between the channels (right). Each channel is normalized to the postinjection signal after injection of FNIR-1072-Dex. **g**, Real-time 4 channel image-guided resection of the tumor (red), lymph node (green) and parallel visualization of blood vessels (magenta) in the same MDA-MB-468 orthotopic tumor-bearing mouse displayed in e, using the custom developed software interface. Color scales in channels 1-4 are 0-15000, 0-25000,0-10000 and 1000-20000 counts. See Supplementary Note 4 for detailed acquisition settings.

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**Methods**

**Computational Chemistry.** Full details of the computational chemistry can be found in **Supplementary Note 1**.

**Synthesis.** Methods for chemical synthesis and detailed characterization of all novel compounds can be found in **Supplementary Note 2**.

**Determination of molar absorption coefficients and fluorescence quantum yields.** All spectroscopic measurements were carried out in duplicate at room temperature using 10 mm × 10 mm quartz cuvettes (Hellma GmbH) and high-purity spectroscopic grade solvents. High purity water ( < 55 µS) was obtained using a MilliPore filter system. Methanol (MeOH; Uvasol), Dimethylsulfoxide (DMSO; spectroscopic grade) and Fetal Bovine Serum (FBS) were purchased from Merck. Dichloroethane (DCE; spectroscopic grade) was purchased from Riedel-de Haen. Phosphate buffered saline (PBS 1x) was prepared by dissolving a pre-made salt tablet (Chemsolute, Th. Geyer) in 500 mL of high purity water according to the specifications of the manufacturer. The reference dyes IR140 and IR26, that were previously studied by us,1 were purchased from Lambda Physik and Exciton, respectively.

Absorption measurements were carried out on a calibrated absorption spectrometer Cary 5000 from Varian, Inc using a step width of 1 nm, an integration time of 0.1 s, and a spectral bandwidth of 2 nm. The absorbance spectra are corrected for blank absorption and scattering. The absorbance of the dye solutions used for fluorescence quantum yield determinations was kept below 0.1 to minimize dye aggregation and reabsorption effects.2

Molar absorption coefficients (ε) at the respective absorbance maximum were determined in methanol, PBS (pH 7.4) or 10% FBS/PBS using Beer’s law, from plots of absorbance vs. concentration. Measurements were performed in 10 mm path length quartz cuvettes (Hellma 111-QS), maintained at 25 °C, with absorbance ≤ 0.20

Fluorescence emission measurements were performed with a calibrated spectrofluorometer FLS-920 from Edinburgh Photonics, equipped with a Xenon lamp, double monochromators, and a nitrogen-cooled PMT R5509P from Hamamatsu. A 550 nm cut-on filter was used in the excitation light path. The emission spectra were obtained by averaging 2 to 6 scans of the emission spectra (depending on the signal-to-noise ratio) with a step width of 1 nm, an integration time of 2 s, excitation and emission slit widths of 16 and 8 mm. Magic-angle conditions with the excitation and emission polarizers set to 0° and 54.7°, respectively, were employed to render the measured emission intensities independent of the emission anisotropy of the NIR emitter. All emission spectra were corrected for excitation intensity fluctuations using the signal of a reference detector as well as for blank emission and scattering by subtraction of the solvent spectra and for the wavelength dependence of the instrument’s spectral responsivity utilizing a previously determined emission correction curve.2 The instrument was flushed with nitrogen during the measurements to avoid light absorption from atmospheric water.1

Fluorescence quantum yields were determined relatively to the dyes IR140 and IR26 following a published procedure2 and calculated according to the following equation

$$ϕ\_{x}=ϕ\_{ref}\frac{F\_{x}f\_{ref}\left(λ\_{ex,ref}\right)n\_{x}^{2}}{F\_{ref}f\_{x}\left(λ\_{ex,x}\right)n\_{ref}^{2}}$$

where ** is the fluorescence quantum yield, *F* is the integral photon flux, *f* is the absorption factor at the excitation wavelength *ex*, and *n* is the refractive index of the solvent. The indices *x* and *ref* denote the sample and reference, respectively. For the reference dyes IR140 and IR26, fluorescence quantum yields of 0.20 in DMSO and 0.0011 in DCE were used that were previously measured absolutely with a calibrated and validated integrating sphere setup.3 The absorption factor *f* at the excitation wavelength was obtained by averaging the absorption over the excitation band width. The spectral width of the spectrofluorometer´s excitation monochromator was not considered. An excitation wavelength of *ex* = 780 nm was used for the dyes IR140 and FNIR-866/872, and *ex* = 900 nm was employed for the dyes IR26 and FNIR-1072. The refractive indices that were employed in the calculations were 1.3314 for MeOH, 1.3341 for PBS, 1.337 for 10 wt% FBS in PBS, 1.479 for DMSO, and 1.4448 for DCE. The integrated photon fluxes required for the determination of the number of emitted photons were obtained in the wavelength ranges of 790 nm – 1100 nm for Cy9-860, 970 nm – 1400 nm for Cy9-1070, 790 nm – 1200 nm for IR140, and 990 nm – 1500 nm for IR26.

**Photostability.** DMSO stock solutions were diluted to 2 μM in PBS (50 mM, pH 7.4) in a 96 well plate (Corning black plate, clear bottom, polystyrene, #3603) in quadruplicate. Samples were continuously irradiated using 780 nm and 850 nm LEDs and the 1064 nm laser for IR-800CW, NIR-866 and FNIR-1072, respectively at a power density of 20 mW/cm2 as measured by a power meter. Single point measurements of the λmax of each dye were recorded at 5 min or 10 minute for 1 hour. Experiments were run in quadruplicate and plotted with error bars derived from the standard deviation (R2 = 0.99 in all cases).

**1O2 Quantum Yields.** DMSO stock solutions (10 mM) of each dye and 1,3-Diphenylisobenzofuran (DPBF) were diluted so that the final concentration of dye in the solution was 5 μM and the concentration of DPBF was ~46 μM. The solutions were dispersed in triplicate in a 96 well plate (Corning black plate, clear bottom, polystyrene, #3603) and irradiated with LEDS (780 nm for IR-800CW/ICG, 850 nm for FNIR-866 and 1050 nm for FNIR-1072) power matched at 5 mW/cm2. The disappearance of the DPBF peak (λmax = 411 nm) was monitored after 60s intervals of irradiation and fit with pseudo first-order reaction rate kinetics. The quantum yields of singlet oxygen production (ΦΔ) were calculated as described previously using ICG as a reference (ΦΔ = 0.008).4

**Chemical Stability.** DMSO stock solutions were diluted into 10% FBS/PBS (50 mM, pH 7.4) in a 96 well plate (Corning black plate, clear bottom, polystyrene, #3603) in quadruplicate. The absorbance at λmax was recorded every 30 minutes for 7 h at room temperature and plotted as a percentage of the initial absorbance values.

**Flatfield correction.** Flatfield images were recorded with 785 or 890 nm (~100 mW/cm2) laser excitation on the reverse side of black laser safety material (Thorlabs, BK5). Images were recorded at 500 ms exposure time and normalized. Images were flatfield corrected by dividing the final processed image by the normalized flatfield image. See **Supplementary Fig. 18** for sample flatfield image and correction.

**Image analysis.** For the data analysis in **Figure 2**, images were acquired (typically an average of 50 to 100 frames), with background subtraction (1 ms) enabled to correct for image sensor non-uniformities and detector noise. The resulting images were then normalized by dividing by the exposure time to obtain images that are in the unit counts/ms. Images in the IR-800CW-Pan and FNIR-872-Pan groups were flatfield corrected as described above.

**Determination of Tumor to Background Ratio (TBR).** Regions of interest (ROIs) were drawn over the tumor, liver, and neck region (used as background). ROI size for the background and organ of interest for a particular animal was kept the same. Mean fluorescence intensity (MFI, counts/ms) was used as a comparing parameter. Statistics (unpaired t-test) were carried out using Prism 8.

**Phantom imaging.** DMSO stock solutions (10 mM) were diluted to 5 μM concentration in MeOH, PBS (50 mM, pH 7.4) or 10% FBS/FBS. Images were acquired using power matched (90 mW/cm2) 785 nm, 890 nm and 1064 nm excitation for IR-800CW, FNIR-866 and FNIR-1072, respectively, w/ 1150 nm long pass (LP) filters. All images were normalized to their exposure time and an ROI was drawn in the same place in each tube to determine the mean fluorescence intensity (in counts/ms).

**Panitumumab conjugation and purification.** Panitumumab-fluorophore conjugates were prepared as described previously.5,6 Briefly, 50 μL of Panitumumab (20 mg/mL commercial stock solution) was added to 25 μL of PBS (1 M, pH 8.5 or 50 mM, pH 7.4)) in a 1.5 mL microcentrifuge tube. In a separate microcentrifuge tube, a molar excess of IR-800CW (3-4 eq for DOL 1.7-1.8), FNIR-872 (5-8 eq for DOL 1.1-1.4) or FNIR-1072 (6-10 eq for DOL 1.0-1.8)NHS esters(as 10 mM stock solutions) were diluted into 25 μL PBS (1 M, pH 8.5 or 50mM, pH 7.4). For the FNIR-1072-antibody conjugation, 3.2 μL DMSO were also added to the solution for a final ca 10% v/v DMSO concentration). The diluted dye solution was immediately transferred to the Panitumumab solution, vortexed and incubated at room temperature in the dark for 1 h. The resulting solutions were eluted through a Zeba spin desalting column (7 kDa MWCO, Thermo Fisher Scientific) to remove unreacted free dye and their absorption spectra directly acquired. The Fluorophore-antibody conjugate solutions were then dialyzed against PBS (50 mM, pH 7.4) using mini-dialysis devices (7 kDa MWCO, Thermo Fisher Scientific) for 3 h at room temperature, and then overnight following exchange for fresh buffer. The antibody conjugate solutions were filtered through a 0.22 μm sterile filter (Acrodisc) and stored at 4 °C. Antibody fluorophore conjugates were used for animal studies within 1 week after labeling.

**Spectral analysis of fluorophore antibody conjugates.** Conjugates of IR-800CW, FNIR-872 and FNIR-1072 were analyzed and DOL calculated as described previously according to the equation below. The conjugates were diluted between 10-40-fold into 1/1 vol/vol MeOH:PBS (50 mM, pH 7.4) and their absorption spectra were recorded on a spectrometer Perkin Elmer Lamba 1050 Plus spectrometer or a Biotek Synergy Mx plate reader using a 384 well plate transparent in the 280 nm range (i.e. UV Co-Star μclear 384 well plate from Greiner Bio-One). The absorption values at 280 nm (A280) and at the cyanine λmax (774, 872 or 1072 nm) were obtained, and the relative dye and antibody concentrations were determined from Beer’s law (C = A/εl). The values obtained were ε = 270,000 M-1 cm-1 for IR-800CW (Li-Cor protein label kit), ε = 240,000 M-1 cm-1 for FNIR-872, ε = 80,000 M-1 cm-1 for FNIR-1072 and εantibody = 203,000 M-1 cm-1 for Panitumumab. A correction factor (CF) of 0.03, 0.06 or 0.23 for IR-800CW, FNIR-872 or FNIR-1072, respectively was applied to account for the absorption contribution of the dyes at 280 nm relative to the λmax. The degree of labeling (DOL), the average number of dye molecules per antibody, was determined from the quotient of dye concentration to antibody concentration. IR-800CW-Panitumumab was prepared and the DOL calculated as described previously.5 The antibody conjugate solutions were filtered through a 0.22 μm sterile filter (Acrodisc) and stored at 4 °C.

$$DOL= \frac{(\frac{A\_{λmax}}{ε\_{dye}})}{(A\_{280 nm}-CF × A\_{λmax})/ε\_{protein}}$$

$$\left[protein\right]\left(\frac{mg}{mL}\right)= \frac{\left(A\_{280 nm}-CF × A\_{λmax}\right)}{ε\_{protein}} × MW\_{protein} × dilution factor$$

**Amino-dextran conjugates.** For full details of the synthesis, purification of Fluorophore-Dextran conjugates,see **Supplementary Note 2**.

**Cell culture.** MDA-MB-468 (EGFR overexpression) human breast cancer cell line was obtained from NCI DTP, DCTD Tumor Repository. The cells were cultured in DMEM supplemented with 2 mM L-glutamine, 11 mM D-glucose, 24 mM sodium bicarbonate, 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. The cells were grown at 37 °C in an atmosphere of 20% O2 and 5% CO2 and were passaged following trypsinization with 0.25% Trypsin-EDTA in PBS. The cells were evaluated for molecular testing of biological materials by animal health diagnostic laboratory at Frederick National Laboratory for Cancer Research.

 **General animal procedures.** *In vivo* studies were performed according to the Frederick National Laboratory for Cancer Research (Frederick, MD, USA) and Helmholtz Zentrum Munich (Munich, BY, Germany) Animal Care and Use committee guidelines. All relevant ethics and protocols for the studies done at Helmholtz Zentrum Munich are in accordance with regulations of the government of Upper Bavaria. Frederick National Laboratory and Helmholtz Zentrum Munich are accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals.

Animal care was provided in accordance with the procedures outlined in the “Guide for Care and Use of Laboratory Animals” (National Research Council; 2011; National Academies Press; Washington, D.C.). Heated pads were located under the anesthesia induction chamber, imaging table, and post procedure recovery cage. Mice body temperature was maintained during the imaging studies except when irradiated with the laser, during which time the heating pad was switched off. For in vivo experiments conducted at Frederick National Laboratory for Cancer Research, the temperature of the mouse did not exceed 40 °C during the imaging experiments, as determined by a handheld infrared camera (FLIR C3). All mice were anesthetized in the induction chamber with 3% isoflurane with filtered (0.2 μm) air at 1 liter/minute flow rate for 3-4 minutes and then modified for imaging to 2% with O2 as a carrier with a flow rate of 1 liter/minute.

**Animal Tumor models.** *MDA-MB-468 Xenograft:* 5-week old female athymic nude mice were purchased from Charles River Laboratories International, Inc. (Frederick, MD). 5 x 106 human breast cancer cells (MDA-MB-468) in 100 μL of Hanks Balanced Salt Solution (HBSS) were subcutaneously injected into the right flank of the mice. Tumors were monitored daily until they reached 4-6 mm in the longest diameter*. In vivo* studies were initiated 10 days post cell injection of the mice.

*Hep3B Liver Orthotopic:*75-week old female athymic nude mice were purchased from Charles River Laboratories International, Inc. (Frederick, MD). Female Athymic nude mice were anesthetized with isoflurane and sterile ophthalmic lubricant was applied to the eyes. The animal was positioned in dorsal recumbency on a flat surface that was kept warm during the procedure. The thoracic and peritoneal regions of skin were prepared aseptically by alternating betadine and ethanol scrubs, repeating three times. A 1-2 cm midline abdominal incision was made starting just below the xiphoid process through the skin and peritoneum to expose the left lobe of the liver. If necessary, the liver lobes were manipulated with an atraumatic instrument such as a moistened sterile cotton swab. A Hamilton syringe with 28G or smaller needle was inserted into the left liver lobe and advanced a few millimeters beyond the puncture site along a subcapsular plane. A volume of 2 μL of 1 × 106 Hep3B cell suspension in HBSS: Matrigel (1:1) was slowly injected into the exposed lobe. Following injection, gentle pressure was applied to injection site for up to 5 minutes with a sterile cotton swab to prevent backflow and to minimize bleeding. The injection site was inspected thoroughly prior to replacing liver into abdomen. The peritoneal layer was closed with absorbable 5-0 suture and skin closed with wound clips. 0.25% Bupivacaine was applied to the skin incision. The animal was provided with a subcutaneous injection of 0.1mg/kg buprenorphine HCl for pain management. Following surgery, the animal recovered in draft free area in a clean cage placed on a warming pad and returned to original cage when animal was alert and active. The mice were monitored weekly by ultrasound until a tumor mass was observed, then biweekly until the tumors reached a size of 800 – 1000 mm3 (average time ~28 days post-surgical implantation).

*MDA-MB-468 Mammary Fatpad Orthotopic:* MDA-MB-468 cells were purchased from ATCC (ATCC number: HTB-132) cultivated in Leibovitz's L­15 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin. The cells were grown at 37 °C in an atmosphere of 20% O2 and 5% CO2and were passaged following trypsinization with 0.25% Trypsin-EDTA in PBS. For the transplantation, ca 7-week old female athymic nude mice purchased from Charles River were used. 3 × 106 human breast cancer cells (MDA-MB-468) were resuspended in 50 μL of Dulbecco’s PBS and orthotopically injected into the mammary fat pad of the mice. Tumors were monitored every two days until they reached 5-7 mm in the longest diameter*. In vivo* studies were initiated ca 30 days post cell injection of the mice.

**Microscopy.** The fluorescence images were acquired using an Olympus IX83-P2ZF inverted widefield microscope. For the excitation of IR-800CW and FNIR-872 a 785nm diode laser (Lumics, LuOcean Mini4) was attenuated and coupled into a liquid light guide (Thorlabs, LGG3-4Z) that was connected to the upper illumination back port of the microscope. An 800 nm short pass filter (Thorlabs FESH0800) was used to clean up the laser, followed by 805 nm dichroic mirror (Thorlabs, DMLP805R). The sample was imaged with an Olympus UPLXAPO 20X objective and a 800nm long pass (Thorlabs, FELH0800) was used as an emission filter. Images were recorded with a Photometrics Prime BSI camera mounted to the trinocular tubus of the microscope.

DAPI was excited with a 365nm LED coupled to a liquid light guide (CoolLED, pE-4000) that was connected to the lower illumination back port of the microscope. The filter configuration used was 377/50nm band pass for cleanup (AHF, F39-377), 409 nm dichroic mirror (AHF, F38-409) and a 447/60 nm band pass (AHF, F37-447) as emission filter. An Olympus UPLXAPO 4X and UPLXAPO 20X objective were used for DAPI together with a Photometrics Prime BSI camera mounted to the trinocular tubus.

**Data Availability**

Image datasets, including all raw and processed imaging data generated in this work, are available at BioImage Archive (accession number xxxx). All other data sets are either included in the supplementary information or as source data files.

**Code Availability**

Custom code (ccda-v3.0.1beta) used to collect data in this work is available at GitHub (<https://gitlab.com/brunslab/ccda>).

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