Supplementary Information

Endocrine-Specific NIR Fluorophores for Adrenal Gland Targeting

Yoshitomo Ashitate[1,2*], Andrew Levitz[3*], Min Ho Park[1,4*], Hoon Hyun[1,5], Vivek Venugopal[1], GwangLi Park[1,6], Georges El Fakhri[6], Maged Henary[3,**], Sylvain Gioux[1,7], John V. Frangioni[1,8], and Hak Soo Choi[1,6,**]

*These authors contributed equally to this work.

[1] Division of Hematology/Oncology, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02215, USA

[2] Department of Gastroenterological Surgery II, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan

[3] Department of Chemistry, Georgia State University, Atlanta, GA 30303, USA

[4] Department of Surgery, Chonnam National University Medical School, Gwangju 501-746, South Korea

[5] Department of Biomedical Sciences, Chonnam National University Medical School, Gwangju 501-746, South Korea

[6] Gordon Center for Medical Imaging, Division of Nuclear Medicine and Molecular Imaging, Department of Radiology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, USA

[7] ICube Laboratory, University of Strasbourg, Illkirch cedex 67412, France

[8] Curadel, LLC, 377 Plantation Street, Worcester, MA 01605 USA

**Correspondence to: M.H. (mhenary1@gsu.edu) or H.S.C. (hchoi12@mgh.harvard.edu)

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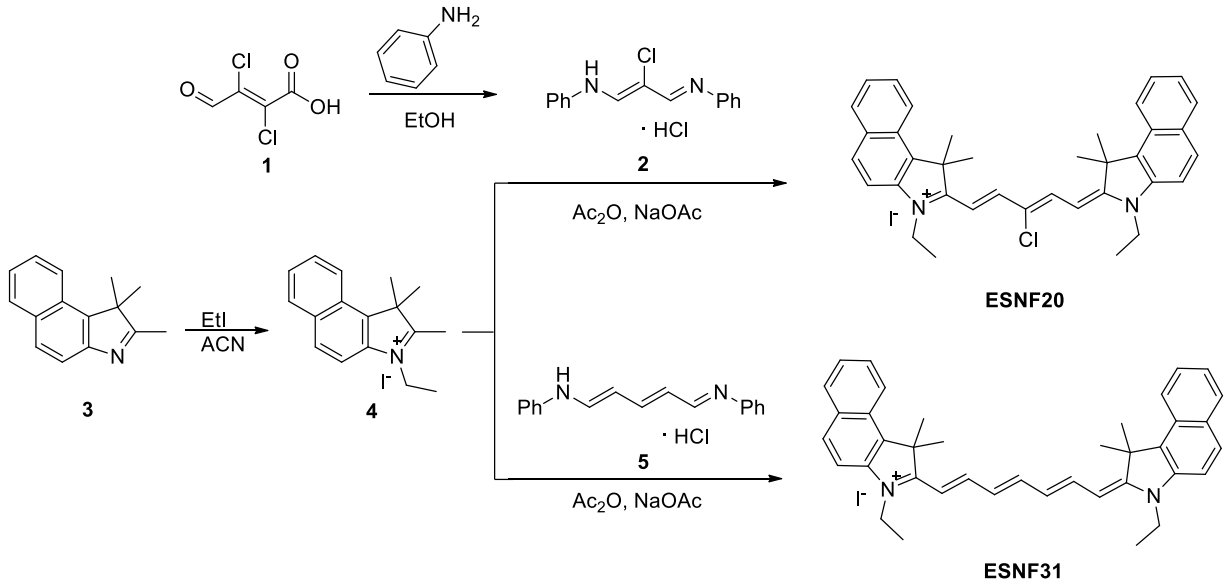
Supplementary Methods

Materials: All chemicals and solvents were of American Chemical Society grade or HPLC purity and were used as received. All chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA), Sigma-Aldrich (Saint Louis, MO), and Acros Organics. The reactions were followed using silica gel 60 F254 thin layer chromatography plates (Merck EMD Millipore, Darmstadt, Germany). The ^{1}H NMR and ^{13}C NMR spectra were obtained using high quality Kontes NMR tubes (Kimble Chase, Vineland, NJ) rated to 500 MHz and were recorded on a Bruker Avance (400 MHz) spectrometer using DMSO-d_6. NMR abbreviations used throughout the experimental section are as follows: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, dd = doublet doublets, and bs = broad singlet. High resolution mass spectra (HRMS) were obtained at the GSU Mass Spectrometry Facility using a Waters Q-TOF micro (ESI-Q-TOF) mass spectrometer. The purity of each compound tested was determined by using LC/MS instrument possessing a Waters 2487 single wavelength absorption detector. The column used in LC was a Waters Delta-Pak 5 µM 100Å 3.9×150 mm reversed phase C_{18} column, with a flow rate of 1 mL/min employing a 5-100% acetonitrile/water/0.1% formic acid gradient; a SEDEX 75 Evaporative light scattering detection (ELSD) was also utilized in tandem with liquid chromatography to confirm purity.



Scheme S1. Synthetic scheme for **ESNF20** and **ESNF31.**

To obtain dyes **ESNF20** and **ESNF31**, 1,1,2-trimethyl-1H-benzo[e]indole **3** was first alkylated by refluxing with iodoethane overnight in acetonitrile to form quaternary ammonium salt **4**. The salt **4** (2 mol eq.) was then stirred in acetic anhydride followed by addition of sodium acetate (2 mol eq.) and linker **2** or **5** (1 mol eq.). The reaction was heated at 80 °C for 2 h. The reactions were monitored closely using regular phase thin layer chromatography with a mobile phase of DCM/MeOH (99:1) as well as UV-Vis-NIR spectrophotometer with methanol as the solvent to visualize the absorption band at ~650 and ~770. Upon completion of the reaction the mixtures were allowed to cool before the dye was precipitated in diethyl ether. The pure products were obtained after dissolving the dyes in acetonitrile and precipitating with ether.

2-((1E,3Z,5E)-3-chloro-5-(3-ethyl-1,1-dimethyl-1H-benzo[e]indol-2(3H)-ylidene)penta-1,3-dien-1-yl)-3-ethyl-1,1-dimethyl-1H-benzo[e]indol-3-ium iodide (**ESNF20**): Yield 68%, mp 231-233 °C; ^{1}H NMR (400 MHz DMSO-d_6) **δ** : 1.39 (s, 6H), 2.00 (s, 12H), 4.37 (s, 4H), 6.38 (d, J = 16 Hz, 2H), 7.55 (t, 2H), 7.73 (t, 2H), 7.84 (d, J = 8 Hz, 2H), 8.12 (m, 4H), 8.27 (d, J = 4 Hz, 2H) 8.65 (d, J = 16 Hz, 2H); ^{13}C NMR (100 MHz DMSO-d_6) **δ** : 11.81, 25.80, 50.72, 98.52, 111.19, 121.60, 121.71, 124.67, 126.96, 127.37, 129.47, 130.01, 131.15, 133.41, 138.56, 146.10, 174.17; HRMS calcd for $C_{37}H_{38}N_2Cl^+$ m/z 545.2724, obsd 545.2717.

3-ethyl-2-((1E,3E,5E,7E)-7-(3-ethyl-1,1-dimethyl-1H-benzo[e]indol-2(3H)-ylidene)hepta-1,3,5-trien-1-yl)-1,1-dimethyl-1H-benzo[e]indol-3-ium iodide (**ESNF31**): Yield 65%, mp 199-201 °C; ^{1}H NMR (400 MHz DMSO-d_6) **δ** : 1.33 (t, J = 6.8 Hz, 6 H), 1.91 (s, 12 H), 4.26 (q, J = 6.8 Hz, 4 H), 6.44 (d, J = 13.2 Hz, 2 H), 6.59 (t, J = 11.6 Hz, 2 H), 7.50 (t, J = 7.2 Hz, 2 H), 7.65 (t, J = 7.6 Hz, 2 H), 7.73 (d, J = 8.8 Hz, 2 H), 7.82 (t, J = 13.2 Hz, 1 H), 7.99 (t, J = 12.4 Hz, 2 H), 8.06 (t, J = 13.2 Hz, 2 H), 8.25 (d, J = 8.4 Hz, 2 H); ^{13}C NMR (100 MHz DMSO-d_6) **δ** : 13.03, 27.17, 50.91, 103.62, 111.83, 122.67, 125.16, 128.13, 130.36, 130.82, 131.70, 133.69, 139.89, 150.53, 172.44; HRMS calcd for $C_{39}H_{41}N_2^+$ m/z 537.3264, obsd 537.3255.

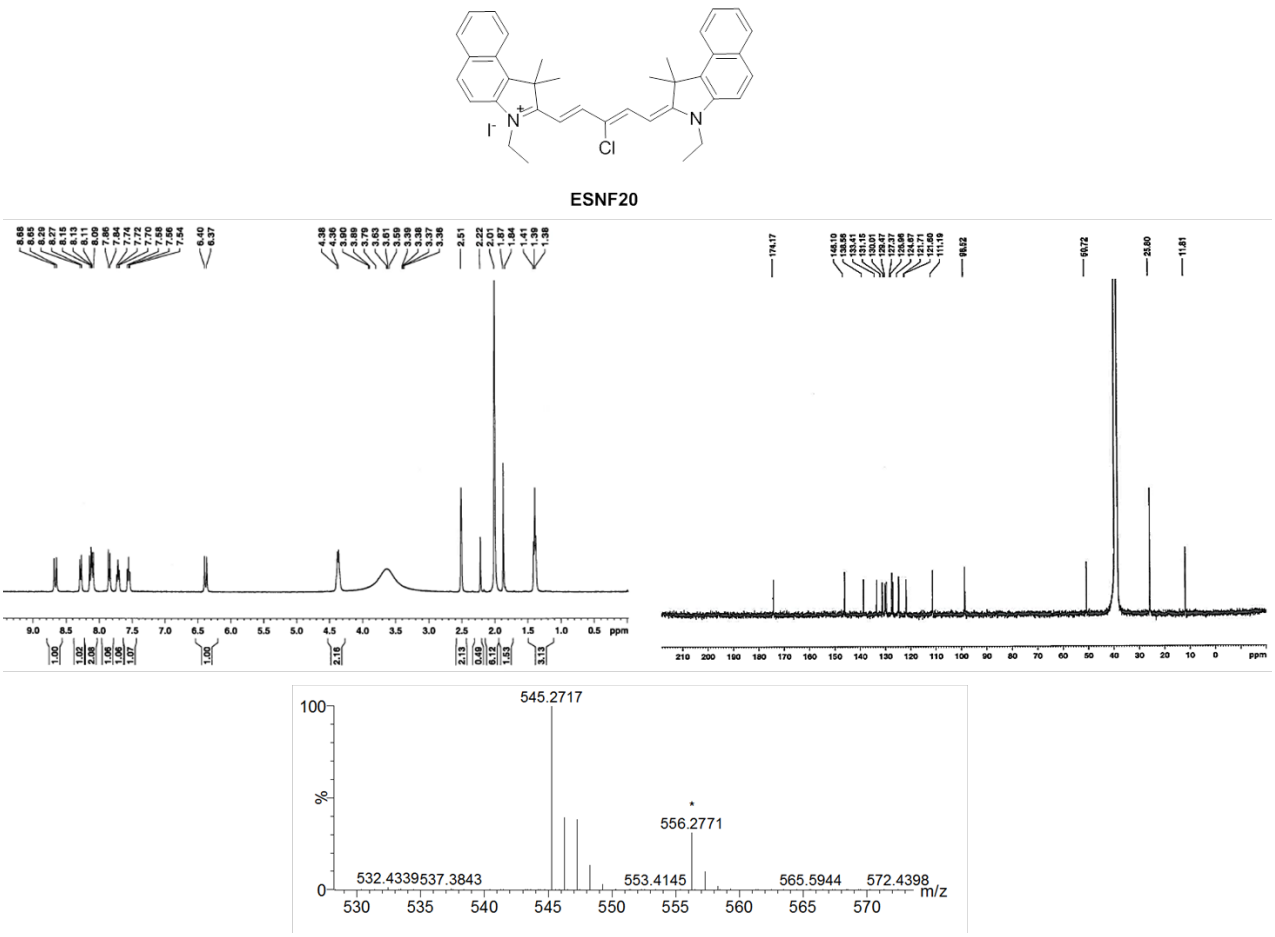


Figure S1. ^{1}H-NMR (top left) and ^{13}C-NMR (top right) of **ESNF20** in DMSO-d_6. ESI-TOF mass spectrum (+) (bottom) was obtained using Waters HPLC-MS with a 5-100% acetonitrile/water/0.1% formic acid gradient.

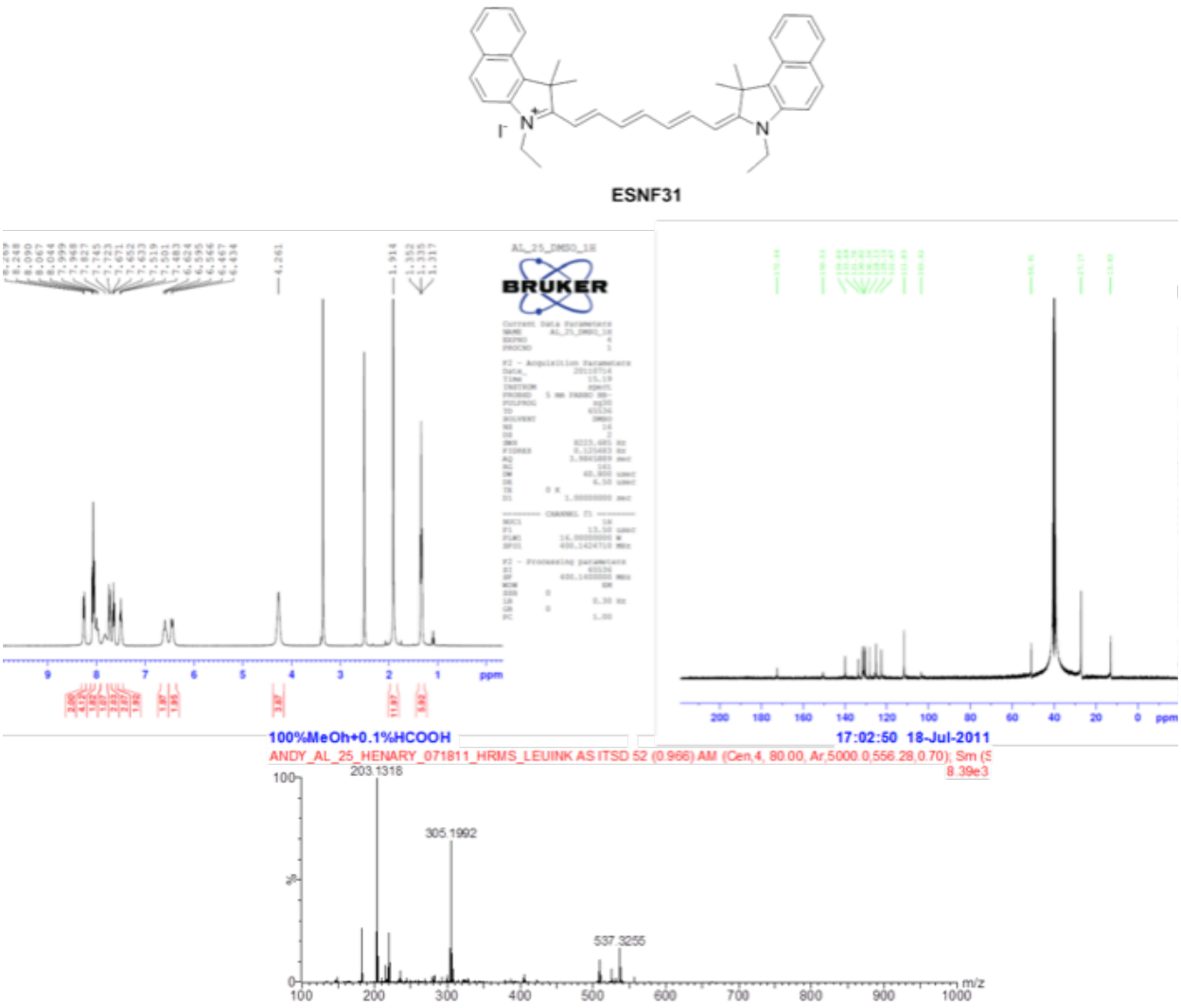


Figure S2. ^{1}H-NMR (top left) and ^{13}C-NMR (top right) of **ESNF31** in DMSO-d_6. ESI-TOF mass spectrum (+) (bottom) was obtained using Waters HPLC-MS with a 5-100% acetonitrile/water/0.1% formic acid gradient.

Optical and physicochemical property analyses: All optical measurements were performed in phosphate-buffered saline (PBS), pH 7.4, 100% fetal bovine serum (FBS) supplemented with 50 mM HEPES, pH 7.4, methanol (MeOH), and dimethyl sulfoxide (DMSO) after preparing 5 mM stock solutions in DMSO. Samples were also diluted in saline containing 10% FBS to confirm aggregation and precipitation in solvent mixtures. Absorbance and fluorescence emission spectra of the series of NIR fluorophores were measured using fiber optic HR2000 absorbance (200–1100 nm) and USB2000FL fluorescence (350–1000 nm) spectrometers (Ocean Optics, Dunedin, FL). NIR excitation was provided by 5 mW of 655 nm red laser pointer (Opcom Inc., Xiamen, China) and 8 mW of 765 nm NIR laser diode light source (Electro Optical Components, Santa Rosa, CA) coupled through a 300 μm core diameter, NA 0.22 fiber (Fiberguide Industries, Stirling, NJ). For fluorescence quantum yield (QY) measurements, oxazine 725 in ethylene glycol (QY = 19%) and ICG in DMSO (QY = 13%) were used as calibration standards, under conditions of matched absorbance at 655 and 765 nm. *In silico* diffusion coefficient (logD at pH 7.4) was calculated using Marvin and JChem calculator plugins (ChemAxon, Budapest, Hungary).

Table S1. Solubility and optical properties of **ESNF20** and **ESNF31** in PBS, FBS, MeOH, and DMSO.

Fluorophore	Solvent	Solubility (mg/mL)	Extinction Coefficient ($M^{-1}cm^{-1}$)	Absorbance Maximum (nm)	Emission Maximum (nm)	Stokes Shift (nm)	Quantum Yield (%)
ESNF20	PBS	< 0.01	63,000	706	720	14	0.59
	FBS	0.01	179,000	692	707	15	16.0
	MeOH	1.80	234,000	681	701	20	6.50
	DMSO	28.0	191,000	686	706	20	10.0
ESNF31	PBS	< 0.01	48,000	775	789	14	5.0
	FBS	0.01	135,000	798	814	16	11.0
	MeOH	16.0	204,000	782	809	27	14.0
	DMSO	39.0	155,000	793	820	27	19.0

DMSO = dimethyl sulfoxide; FBS = fetal bovine serum; MeOH = methanol; PBS = phosphate-buffered saline.

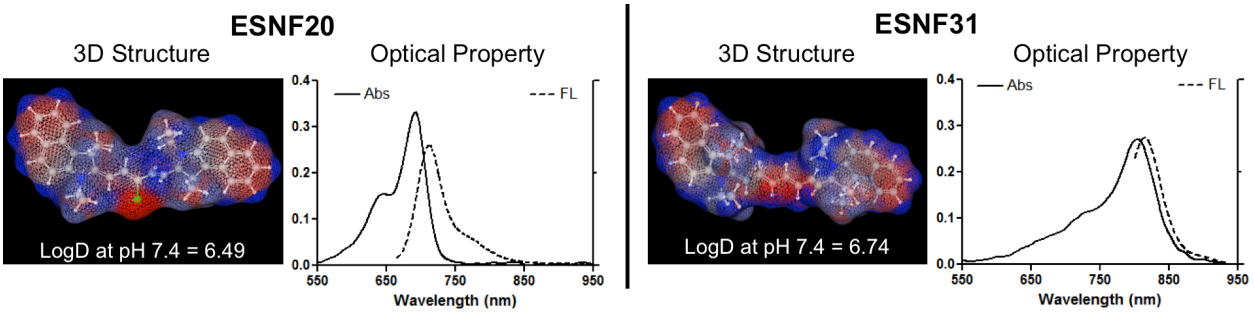


Figure S3. 3D structures and optical properties of **ESNF20** and **ESNF31**. Representative absorbance (Abs) and fluorescence (FL) spectra of each fluorophore were measured in 100% FBS supplemented with 50 mM HEPES, pH 7.4. LogD was calculated using MarvinSketch (ChemAxon).

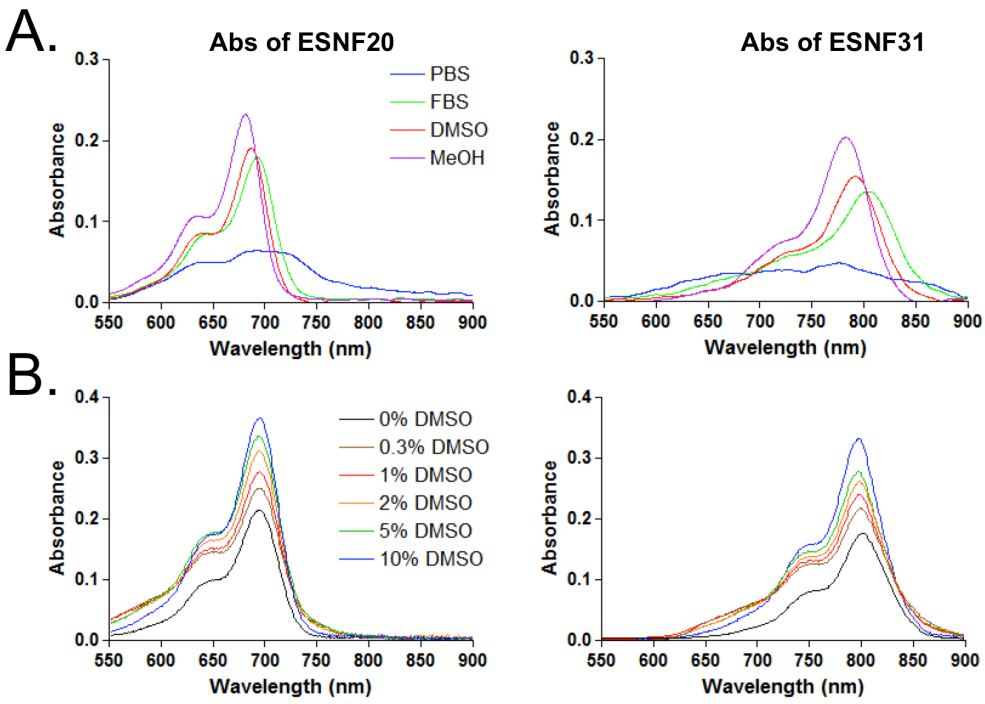


Figure S4. Absorbance spectra of **ESNF20** and **ESNF31** in various solvents. (A) Absorbance spectra in PBS, FBS, DMSO and MeOH. (B) Solubility in saline-DMSO mixtures. 5 mM tock solutions were prepared in DMSO and added to saline containing 10% FBS, followed by centrifugation to confirm aggregation and precipitation.

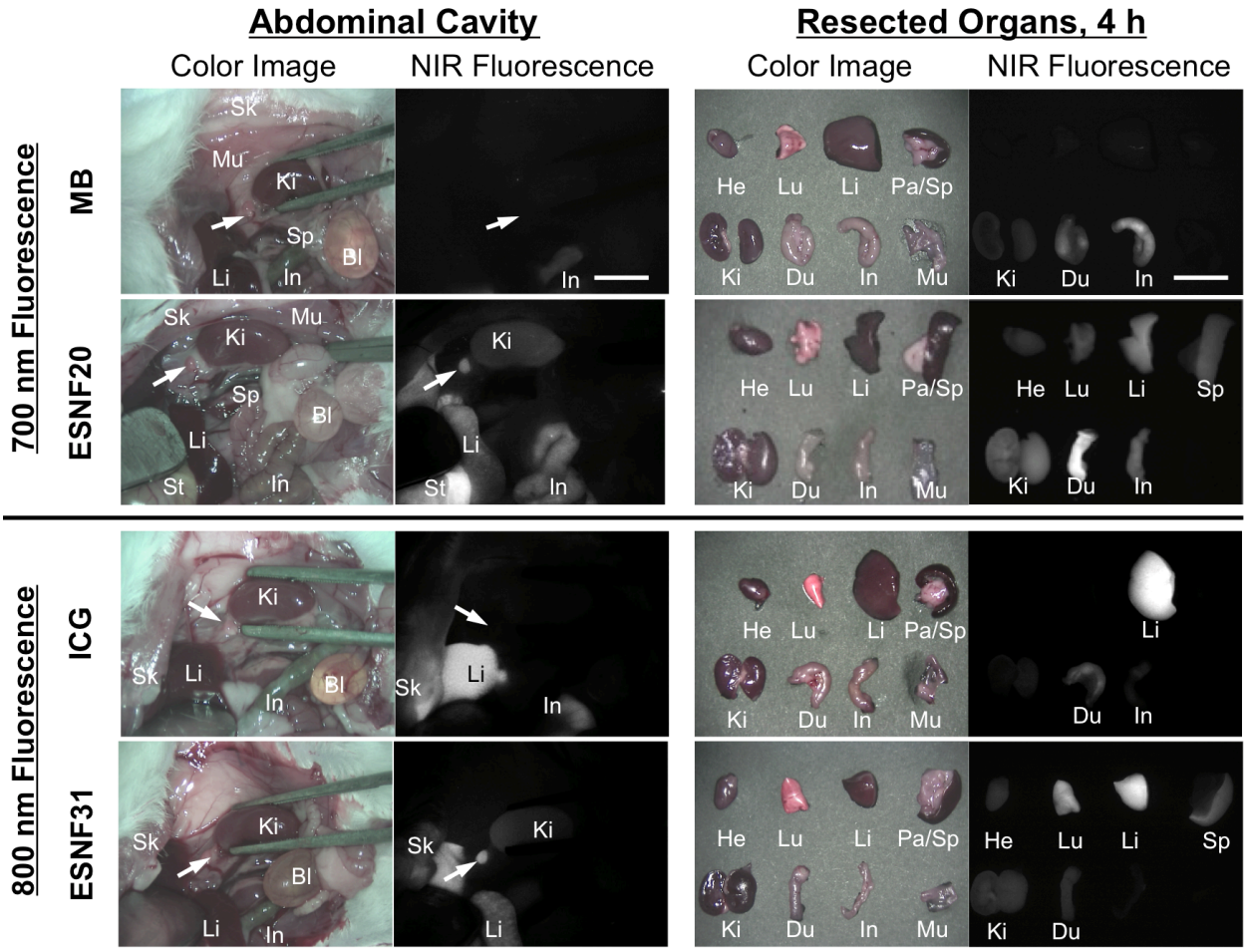


Figure S5. *In vivo* biodistribution of MB, **ESNF20**, ICG, and **ESNF31** in mice. 10 nmol of each fluorophore was injected intravenously into 20 g CD-1 mice (0.2 mg/kg) 4 h prior to imaging. Abbreviations used are: Bl, bladder; Du, duodenum; He, Heart; In, intestine; Ki, kidneys; Li, liver; Lu, lungs; Mu, muscle; Pa, pancreas; Sk, skin; Sp, spleen; St, stomach. Arrows indicate adrenal glands. Scale bars = 1 cm.