

## Supplementary Information

### Near infrared fluorescent nanoparticles derived from hyaluronic acid improve tumor contrast for image-guided surgery

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#### Supplementary Methods

##### *Dye Release*

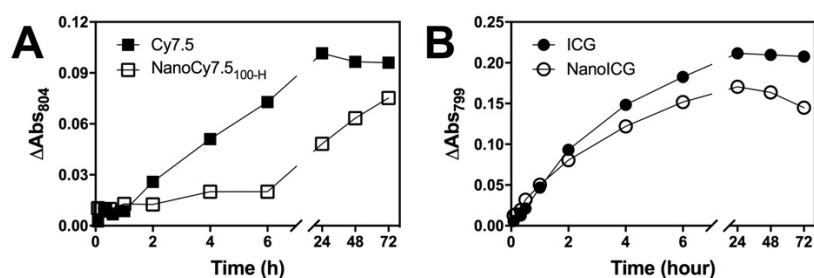
Release of ICG and Cy7.5 from their respective HA NP formulations (lead NPs based on contrast enhancement studies) was determined in release media, which is constituted from BSA dissolved in PBS to 42.5 mg/ml BSA. NanoICG<sub>PBA</sub>, ICG, NanoCy7.5<sub>100-H</sub>, or Cy7.5, were dissolved in release media to 20  $\mu$ M, 20  $\mu$ M, 13.8  $\mu$ M, and 20  $\mu$ M, respectively, based on dye content. Release of free ICG and Cy7.5 from NPs relative to dye alone was examined via dialysis. Solutions containing NP dye formulation or dye alone (1 ml) was placed in 100 kDa MWCO dialysis tubing and immersed in 10 mL of release media in 15 ml conical tubes, sealed, and placed on a rocker. Dye released into release media was measured at 5, 10, 20, and 30 min and 1, 2, 4, 6, 24, 48, and 72 h by taking 600  $\mu$ l from the conical tube and absorbance spectra were obtained with a 220 UV-Vis Spectrophotometer (Thermo Scientific). Release media was subtracted using Thermo

INSIGHT software. After spectra acquisition the sample was returned to the tube. Release of dye from NP was and transport of free dye was measured as appearance of dye in the release media outside the dialysis tubing. For ICG and NanoICG<sub>PBA</sub>, this was measured as  $\Delta\text{abs}_{799} = \text{abs}_{799}$  aliquot –  $\text{abs}_{799}$  release media, while for Cy7.5 and NanoCy7.5,  $\Delta\text{abs}_{804} = \text{abs}_{804}$  aliquot –  $\text{abs}_{804}$  release media.

## Supplementary Results

### *Dye Release*

The release of Cy7.5 or ICG from NP formulations was compared to the respective dye alone. Albumin was required both inside and outside the bag due to the poor solubility the NIR dyes. The presence of serum albumin also more accurately reflects the conditions that the NPs developed in the work interact with upon systemic administration. Supplemental Figure 1 shows that both free Cy7.5 and ICG had rapid transport across the dialysis membrane via albumin with the majority of peak dye concentration occurring within the first 6 hours. HA-Cy7.5 conjugate more slowly released compared to Cy7.5, which is likely due to direct chemical conjugation of Cy7.5 to HA. ICG that as physically entrapped in HA-based NPs rapidly released into albumin. It is not likely that NanoICG diffused across the membrane. We have previously reported that ICG entrapped in HA NPs has broad scattering due to close packing [26]. The ICG spectra collected from the conical tubes is consistent with albumin bound ICG [36].



**Supplemental Figure 1.** Release of Cy7.5 and ICG from NP formulations. (A) NanoCy7.5<sub>100-H</sub> (open squares) and Cy7.5 (filled squares). (B) NanoICG<sub>PBA</sub> (open circles) and ICG (filled circles).



Tumor									
ICG vs. NanoICG <sub>PBA</sub>	34.9	18.29	ns	6.95	15.9	****	4.624	14.43	****
ICG vs. NanoICG <sub>5bCA</sub>	34.9	16.1	ns	6.95	14.2	****	4.624	9.167	***
NanoICG <sub>PBA</sub> vs. NanoICG <sub>5bCA</sub>	18.29	16.1	ns	15.9	14.2	ns	14.43	9.167	****
Muscle									
ICG vs. NanoICG <sub>PBA</sub>	22.1	4.984	ns	2.13	2.13	ns	0.653	1.628	ns
ICG vs. NanoICG <sub>5bCA</sub>	22.1	10.6	ns	2.13	2.78	ns	0.653	1.711	ns
NanoICG <sub>PBA</sub> vs. NanoICG <sub>5bCA</sub>	4.984	10.6	ns	2.13	2.78	ns	1.628	1.711	ns
Lung									
ICG vs. NanoICG <sub>PBA</sub>	43	23.18	ns	10.3	9.41	ns	2.281	7.017	*
ICG vs. NanoICG <sub>5bCA</sub>	43	23.3	ns	10.3	9.77	ns	2.281	7.202	**
NanoICG <sub>PBA</sub> vs. NanoICG <sub>5bCA</sub>	23.18	23.3	ns	9.41	9.77	ns	7.017	7.202	ns
Heart									
ICG vs. NanoICG <sub>PBA</sub>	18.8	13.67	ns	3.65	5.04	ns	1.518	3.958	ns
ICG vs. NanoICG <sub>5bCA</sub>	18.8	9.73	ns	3.65	7.19	ns	1.518	4.1	ns
NanoICG <sub>PBA</sub> vs. NanoICG <sub>5bCA</sub>	13.67	9.73	ns	5.04	7.19	ns	3.958	4.1	ns
Bone									
ICG vs. NanoICG <sub>PBA</sub>	18.9	15.07	ns	7.23	10	ns	3.393	7.547	*
ICG vs. NanoICG <sub>5bCA</sub>	18.9	12.7	ns	7.23	9.52	ns	3.393	8.472	**
NanoICG <sub>PBA</sub> vs. NanoICG <sub>5bCA</sub>	15.07	12.7	ns	10	9.52	ns	7.547	8.472	ns
Kidney									
ICG vs. NanoICG <sub>PBA</sub>	177	54.55	****	21.4	23.3	ns	10.11	30.36	****
ICG vs. NanoICG <sub>5bCA</sub>	177	74.2	****	21.4	20.8	ns	10.11	30.48	****
NanoICG <sub>PBA</sub> vs. NanoICG <sub>5bCA</sub>	54.55	74.2	ns	23.3	20.8	ns	30.36	30.48	ns
Pancreas									
ICG vs. NanoICG <sub>PBA</sub>	49	20.71	ns	7.63	7.98	ns	1.945	5.846	*
ICG vs. NanoICG <sub>5bCA</sub>	49	35.4	ns	7.63	7.58	ns	1.945	6.33	*
NanoICG <sub>PBA</sub> vs. NanoICG <sub>5bCA</sub>	20.71	35.4	ns	7.98	7.58	ns	5.846	6.33	ns

Liver									
ICG vs. NanoICG <sub>PBA</sub>	95	100.3	ns	21.2	53.2	****	7.405	32.13	****
ICG vs. NanoICG <sub>5bCA</sub>	95	73.7	ns	21.2	40.4	****	7.405	37.08	****
NanoICG <sub>PBA</sub> vs. NanoICG <sub>5bCA</sub>	100.3	73.7	ns	53.2	40.4	****	32.13	37.08	**
Spleen									
ICG vs. NanoICG <sub>PBA</sub>	28.8	19.75	ns	9.14	16.3	**	3.643	15.71	****
ICG vs. NanoICG <sub>5bCA</sub>	28.8	17.7	ns	9.14	14	ns	3.643	17.19	****
NanoICG <sub>PBA</sub> vs. NanoICG <sub>5bCA</sub>	19.75	17.7	ns	16.3	14	ns	15.71	17.19	ns

NanoCy7.5 <sub>10</sub>	4 h			24 h			72 h		
	Y1	Y2	Sig.	Y1	Y2	Sig.	Y1	Y2	Sig.
Tumor									
Cy7.5 vs. NanoCy7.5 <sub>10-Ø</sub>	11.81	51.23	ns	13.55	55.71	ns	21.88	42.77	ns
Cy7.5 vs. NanoCy7.5 <sub>10-L</sub>	11.81	24.28	ns	13.55	45.44	ns	21.88	109.3	***
Cy7.5 vs. NanoCy7.5 <sub>10-H</sub>	11.81	68.25	ns	13.55	70.08	ns	21.88	72.36	ns
NanoCy7.5 <sub>10-Ø</sub> vs. NanoCy7.5 <sub>10-L</sub>	51.23	24.28	ns	55.71	45.44	ns	42.77	109.3	*
NanoCy7.5 <sub>10-Ø</sub> vs. NanoCy7.5 <sub>10-H</sub>	51.23	68.25	ns	55.71	70.08	ns	42.77	72.36	ns
NanoCy7.5 <sub>10-L</sub> vs. NanoCy7.5 <sub>10-H</sub>	24.28	68.25	ns	45.44	70.08	ns	109.3	72.36	ns
Muscle									
Cy7.5 vs. NanoCy7.5 <sub>10-Ø</sub>	23.8	29.63	ns	12.18	16.85	ns	18.89	9.338	ns
Cy7.5 vs. NanoCy7.5 <sub>10-L</sub>	23.8	29.78	ns	12.18	29.45	ns	18.89	15.77	ns
Cy7.5 vs. NanoCy7.5 <sub>10-H</sub>	23.8	45	ns	12.18	33.11	ns	18.89	15.7	ns
NanoCy7.5 <sub>10-Ø</sub> vs. NanoCy7.5 <sub>10-L</sub>	29.63	29.78	ns	16.85	29.45	ns	9.338	15.77	ns
NanoCy7.5 <sub>10-Ø</sub> vs. NanoCy7.5 <sub>10-H</sub>	29.63	45	ns	16.85	33.11	ns	9.338	15.7	ns
NanoCy7.5 <sub>10-L</sub> vs. NanoCy7.5 <sub>10-H</sub>	29.78	45	ns	29.45	33.11	ns	15.77	15.7	ns
Lung									
Cy7.5 vs. NanoCy7.5 <sub>10-Ø</sub>	112.6	251.8	*	67.21	52.78	ns	68.06	30.71	ns
Cy7.5 vs. NanoCy7.5 <sub>10-L</sub>	112.6	202.2	ns	67.21	85.96	ns	68.06	70.83	ns
Cy7.5 vs. NanoCy7.5 <sub>10-H</sub>	112.6	325.5	***	67.21	95.18	ns	68.06	64.26	ns
NanoCy7.5 <sub>10-Ø</sub> vs. NanoCy7.5 <sub>10-L</sub>	251.8	202.2	ns	52.78	85.96	ns	30.71	70.83	ns



NanoCy7.5 <sub>10-∅</sub> NanoCy7.5 <sub>10-H</sub>	vs.	130.5	175	ns	44.38	76.58	ns	48.8	66.64	ns
NanoCy7.5 <sub>10-L</sub> NanoCy7.5 <sub>10-H</sub>	vs.	95.09	175	ns	72.75	76.58	ns	80.88	66.64	ns
Liver										
Cy7.5 vs. NanoCy7.5 <sub>10-∅</sub>		1432	1128	****	834.6	1270	****	474.5	865.2	****
Cy7.5 vs. NanoCy7.5 <sub>10-L</sub>		1432	1602	**	834.6	1217	****	474.5	1264	****
Cy7.5 vs. NanoCy7.5 <sub>10-H</sub>		1432	1715	****	834.6	1468	****	474.5	1098	****
NanoCy7.5 <sub>10-∅</sub> NanoCy7.5 <sub>10-L</sub>	vs.	1128	1602	****	1270	1217	ns	865.2	1264	****
NanoCy7.5 <sub>10-∅</sub> NanoCy7.5 <sub>10-H</sub>	vs.	1128	1715	****	1270	1468	***	865.2	1098	****
NanoCy7.5 <sub>10-L</sub> NanoCy7.5 <sub>10-H</sub>	vs.	1602	1715	ns	1217	1468	****	1264	1098	****
Spleen										
Cy7.5 vs. NanoCy7.5 <sub>10-∅</sub>		96.33	225.6	ns	78.27	314	****	46.76	138	**
Cy7.5 vs. NanoCy7.5 <sub>10-L</sub>		96.33	138.7	ns	78.27	149.8	ns	46.76	243.4	****
Cy7.5 vs. NanoCy7.5 <sub>10-H</sub>		96.33	201.6	ns	78.27	223.5	*	46.76	172.1	****
NanoCy7.5 <sub>10-∅</sub> NanoCy7.5 <sub>10-L</sub>	vs.	225.6	138.7	ns	314	149.8	**	138	243.4	***
NanoCy7.5 <sub>10-∅</sub> NanoCy7.5 <sub>10-H</sub>	vs.	225.6	201.6	ns	314	223.5	ns	138	172.1	ns
NanoCy7.5 <sub>10-L</sub> NanoCy7.5 <sub>10-H</sub>	vs.	138.7	201.6	ns	149.8	223.5	ns	243.4	172.1	*

NanoCy7.5 <sub>100</sub>	4 h			24 h			72 h			
	ȳ1	ȳ2	Sig.	ȳ1	ȳ2	Sig.	ȳ1	ȳ2	Sig.	
Test details										
Tumor										
Cy7.5 vs. NanoCy7.5 <sub>100-∅</sub>	11.81	33.98	ns	13.55	18.13	ns	21.88	34.84	ns	
Cy7.5 vs. NanoCy7.5 <sub>100-L</sub>	11.81	63.97	ns	13.55	43.79	ns	21.88	35.99	ns	
Cy7.5 vs. NanoCy7.5 <sub>100-H</sub>	11.81	38.04	ns	13.55	118.4	ns	21.88	34.01	ns	
NanoCy7.5 <sub>100-∅</sub> NanoCy7.5 <sub>100-L</sub>	vs.	33.98	63.97	ns	18.13	43.79	ns	34.84	35.99	ns
NanoCy7.5 <sub>100-∅</sub> NanoCy7.5 <sub>100-H</sub>	vs.	33.98	38.04	ns	18.13	118.4	ns	34.84	34.01	ns
NanoCy7.5 <sub>100-L</sub> NanoCy7.5 <sub>100-H</sub>	vs.	63.97	38.04	ns	43.79	118.4	ns	35.99	34.01	ns
Muscle										
Cy7.5 vs. NanoCy7.5 <sub>100-∅</sub>	23.8	19.3	ns	12.18	7.648	ns	18.89	10	ns	

Cy7.5 vs. NanoCy7.5 <sub>100-L</sub>	23.8	22.61	ns	12.18	25.94	ns	18.89	11.87	ns
Cy7.5 vs. NanoCy7.5 <sub>100-H</sub>	23.8	35.04	ns	12.18	16.45	ns	18.89	9.885	ns
NanoCy7.5 <sub>100-∅</sub> vs. NanoCy7.5 <sub>100-L</sub>	19.3	22.61	ns	7.648	25.94	ns	10	11.87	ns
NanoCy7.5 <sub>100-∅</sub> vs. NanoCy7.5 <sub>100-H</sub>	19.3	35.04	ns	7.648	16.45	ns	10	9.885	ns
NanoCy7.5 <sub>100-L</sub> vs. NanoCy7.5 <sub>100-H</sub>	22.61	35.04	ns	25.94	16.45	ns	11.87	9.885	ns
<b>Lung</b>									
Cy7.5 vs. NanoCy7.5 <sub>100-∅</sub>	112.6	156.8	ns	67.21	27.88	ns	68.06	36.18	ns
Cy7.5 vs. NanoCy7.5 <sub>100-L</sub>	112.6	231.6	ns	67.21	75	ns	68.06	67.72	ns
Cy7.5 vs. NanoCy7.5 <sub>100-H</sub>	112.6	233.3	ns	67.21	84.62	ns	68.06	47.64	ns
NanoCy7.5 <sub>100-∅</sub> vs. NanoCy7.5 <sub>100-L</sub>	156.8	231.6	ns	27.88	75	ns	36.18	67.72	ns
NanoCy7.5 <sub>100-∅</sub> vs. NanoCy7.5 <sub>100-H</sub>	156.8	233.3	ns	27.88	84.62	ns	36.18	47.64	ns
NanoCy7.5 <sub>100-L</sub> vs. NanoCy7.5 <sub>100-H</sub>	231.6	233.3	ns	75	84.62	ns	67.72	47.64	ns
<b>Heart</b>									
Cy7.5 vs. NanoCy7.5 <sub>100-∅</sub>	82.04	79.42	ns	47.51	13.88	ns	49.6	22.84	ns
Cy7.5 vs. NanoCy7.5 <sub>100-L</sub>	82.04	138.9	ns	47.51	56.86	ns	49.6	40.88	ns
Cy7.5 vs. NanoCy7.5 <sub>100-H</sub>	82.04	154.1	ns	47.51	51.25	ns	49.6	28.33	ns
NanoCy7.5 <sub>100-∅</sub> vs. NanoCy7.5 <sub>100-L</sub>	79.42	138.9	ns	13.88	56.86	ns	22.84	40.88	ns
NanoCy7.5 <sub>100-∅</sub> vs. NanoCy7.5 <sub>100-H</sub>	79.42	154.1	ns	13.88	51.25	ns	22.84	28.33	ns
NanoCy7.5 <sub>100-L</sub> vs. NanoCy7.5 <sub>100-H</sub>	138.9	154.1	ns	56.86	51.25	ns	40.88	28.33	ns
<b>Bone</b>									
Cy7.5 vs. NanoCy7.5 <sub>100-∅</sub>	67.04	156.3	ns	44.86	141.7	ns	37.62	165.4	****
Cy7.5 vs. NanoCy7.5 <sub>100-L</sub>	67.04	149.2	ns	44.86	205.6	ns	37.62	140.2	****
Cy7.5 vs. NanoCy7.5 <sub>100-H</sub>	67.04	148.2	ns	44.86	221.8	ns	37.62	144.6	****
NanoCy7.5 <sub>100-∅</sub> vs. NanoCy7.5 <sub>100-L</sub>	156.3	149.2	ns	141.7	205.6	ns	165.4	140.2	ns
NanoCy7.5 <sub>100-∅</sub> vs. NanoCy7.5 <sub>100-H</sub>	156.3	148.2	ns	141.7	221.8	ns	165.4	144.6	ns
NanoCy7.5 <sub>100-L</sub> vs. NanoCy7.5 <sub>100-H</sub>	149.2	148.2	ns	205.6	221.8	ns	140.2	144.6	ns
<b>Kidney</b>									
Cy7.5 vs. NanoCy7.5 <sub>100-∅</sub>	370	253	ns	286.5	78.22	ns	361.6	144.4	****



Cy7.5 vs. NanoCy7.5 <sub>100-L</sub>	370	374.9	ns	286.5	275.5	ns	361.6	226.4	****
Cy7.5 vs. NanoCy7.5 <sub>100-H</sub>	370	313.8	ns	286.5	193.4	ns	361.6	194.9	****
NanoCy7.5 <sub>100-∅</sub> vs. NanoCy7.5 <sub>100-L</sub>	253	374.9	ns	78.22	275.5	ns	144.4	226.4	***
NanoCy7.5 <sub>100-∅</sub> vs. NanoCy7.5 <sub>100-H</sub>	253	313.8	ns	78.22	193.4	ns	144.4	194.9	ns
NanoCy7.5 <sub>100-L</sub> vs. NanoCy7.5 <sub>100-H</sub>	374.9	313.8	ns	275.5	193.4	ns	226.4	194.9	ns
Pancreas									
Cy7.5 vs. NanoCy7.5 <sub>100-∅</sub>	59.98	75.62	ns	45.04	23.05	ns	68.86	41.94	ns
Cy7.5 vs. NanoCy7.5 <sub>100-L</sub>	59.98	116.4	ns	45.04	65.66	ns	68.86	77.27	ns
Cy7.5 vs. NanoCy7.5 <sub>100-H</sub>	59.98	149.5	ns	45.04	48.84	ns	68.86	65.6	ns
NanoCy7.5 <sub>100-∅</sub> vs. NanoCy7.5 <sub>100-L</sub>	75.62	116.4	ns	23.05	65.66	ns	41.94	77.27	ns
NanoCy7.5 <sub>100-∅</sub> vs. NanoCy7.5 <sub>100-H</sub>	75.62	149.5	ns	23.05	48.84	ns	41.94	65.6	ns
NanoCy7.5 <sub>100-L</sub> vs. NanoCy7.5 <sub>100-H</sub>	116.4	149.5	ns	65.66	48.84	ns	77.27	65.6	ns
Liver									
Cy7.5 vs. NanoCy7.5 <sub>100-∅</sub>	1432	1348	ns	834.6	681.1	ns	474.5	858	****
Cy7.5 vs. NanoCy7.5 <sub>100-L</sub>	1432	1288	ns	834.6	1773	****	474.5	1205	****
Cy7.5 vs. NanoCy7.5 <sub>100-H</sub>	1432	1232	*	834.6	1079	*	474.5	1041	****
NanoCy7.5 <sub>100-∅</sub> vs. NanoCy7.5 <sub>100-L</sub>	1348	1288	ns	681.1	1773	****	858	1205	****
NanoCy7.5 <sub>100-∅</sub> vs. NanoCy7.5 <sub>100-H</sub>	1348	1232	ns	681.1	1079	***	858	1041	****
NanoCy7.5 <sub>100-L</sub> vs. NanoCy7.5 <sub>100-H</sub>	1288	1232	ns	1773	1079	****	1205	1041	****
Spleen									
Cy7.5 vs. NanoCy7.5 <sub>100-∅</sub>	96.33	603.3	****	78.27	316.7	*	46.76	263.4	****
Cy7.5 vs. NanoCy7.5 <sub>100-L</sub>	96.33	594.7	****	78.27	703.9	****	46.76	423.8	****
Cy7.5 vs. NanoCy7.5 <sub>100-H</sub>	96.33	795.2	****	78.27	641.8	****	46.76	247.6	****
NanoCy7.5 <sub>100-∅</sub> vs. NanoCy7.5 <sub>100-L</sub>	603.3	594.7	ns	316.7	703.9	***	263.4	423.8	****
NanoCy7.5 <sub>100-∅</sub> vs. NanoCy7.5 <sub>100-H</sub>	603.3	795.2	ns	316.7	641.8	**	263.4	247.6	ns
NanoCy7.5 <sub>100-L</sub> vs. NanoCy7.5 <sub>100-H</sub>	594.7	795.2	*	703.9	641.8	ns	423.8	247.6	****

**Supplemental Table 2.** Values and significance levels for organ SNR in BALB/c mice for lead NP formulations at 4 and 24 h.

NanoICG	4 h	24 h
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	ICG	NanoICG	Sig.	ICG	NanoICG	Sig.
Tumor	53.68	46.46	ns	13.4	29.19	****
Muscle	5.144	6.447	ns	1.179	2.133	ns
Lung	29.05	27.42	ns	5.668	8.295	ns
Heart	10.57	10.7	ns	2.067	4.261	ns
Bone	12.68	17.62	ns	4.811	8.248	ns
Pancreas	24.91	48.86	ns	3.239	6.255	ns
Kidney	108.3	55.95	***	20.48	26.28	**
Liver	142.5	90.04	***	19.08	30.87	****
Spleen	12.07	23.83	ns	4.688	12.39	****

NanoCy7.5	4 h			24 h		
	Cy7.5	NanoCy7.5 <sub>100-H</sub>	Sig.	Cy7.5	NanoCy7.5 <sub>100-H</sub>	Sig.
Tumor	47.77	231.3	***	29.02	270.3	****
Muscle	26.72	43.52	ns	12.5	24.58	ns
Lung	256.2	378.8	ns	73.86	100.8	ns
Heart	74.34	164.5	ns	28.41	42.58	ns
Bone	67.33	116.1	ns	34.02	102.6	*
Pancreas	65.48	150.3	ns	38.6	77.58	ns
Kidney	283.6	361.4	ns	180.6	170.9	ns
Liver	1017	937.5	ns	467.8	744.3	****
Spleen	329.1	347	ns	139.4	404.5	****