## Tuned near infrared fluorescent hyaluronic acid conjugates for delivery to pancreatic cancer for intraoperative imaging

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**Video 3**. Wide-field imaging recorded for the NIRF accumulation of HA20k-IRDye800 in abdominal metastases in PDAC bearing mice.



**Figure S1.** Absorption and fluorescence emission pattern of different molecular weight (MW) of HA conjugated dyes in ultrapure  $H_2O$  and 50/50 DMSO/ $H_2O$ .  $HA_{20k}$ -NIRF conjugates were illustrated in **Figure 1**. The excitation wavelength for absorption ranges from 600 nm to 900 nm, the fluorescence emission was collected between 790 nm to 900 nm. The concentrations of contrast agents were consistent in altered solvents.



Figure **S2**. TEM images of BSA (A), BSA mixed with HA<sub>20k</sub>-Cy7.5 (B), BSA mixed with HA<sub>20k</sub>-IRDye800 (C) with negative staining, scale bar represents 100 nm.



**Figure S3.** Relative biodistribution of HA-Cy7.5 and HA-IRDye800 in WT C57BL/6 mice 24 h post intravenous injection. (**A**, **C**) Quantitative organ biodistribution of HA-Cy7.5 and HA-IRDye800 were determined by the relative fluorescence of each organ from NIRF images, respectively. (**B**, **D**) Representative images of each HA MW<sub>N</sub> and dye in organs of interest in WT C57BL/6 mice, which demonstrate the predominant clearance route of each contrast agent. Images of necropsied organs were acquired with the 800 nm channel of pearl trilogy small animal imaging system, interfering signal from neighboring organs was manually removed. N = 3, <sup>a</sup>p < 0.05 as compared to HA<sub>20k</sub>-dye, <sup>d</sup>p < 0.05 as compared to HA<sub>100k</sub>-dye.



**Figure S4.** The biodistribution of HA-dye conjugates in WT C57BL/6 and pancreatic ductal adenocarcinoma (PDAC)-bearing mice. (A) Organ biodistribution of HA-Cy7.5 in wide type (WT) C57BL/6 mice 96 h post injection; (B) Organ biodistribution of HA-Cy7.5 in PDAC-bearing mice 96 h post injection; (C) Organ biodistribution of HA-IRDye800 in WT C57BL/6 mice 96 h post injection; (D) Organ biodistribution of HA-IRDye800 in PDAC-bearing mice 96 h post injection; signal to noise ratio (SNR) was calculated based of fluorescence of organ divided by the background noise, N = 5. <sup>a</sup>p < 0.05 as compared to free dye, <sup>b</sup>p < 0.05 as compared to HA<sub>5k</sub>-dye, <sup>c</sup>p < 0.05 as compared to HA<sub>20k</sub>-dye, <sup>d</sup>p < 0.05 as compared to HA<sub>100k</sub>-dye.



**Figure S5.** Images from surgical navigation of pancreatic tumor contrast-enhanced with HA-dye 96 h post *i.v.* injection. Livers and spleens were removed due to high background signal from clearance, spleens that completely involved with PDAC were kept. PT = pancreatic tumor, HP = healthy (uninvolved) pancreas, St = stomach, SI = small intestine, Int = large intestine; each group was arranged into three rows: (*Top*) Color images show orthotopic PDAC (PT) and the location of the handheld spectroscopic pen, which provides NIR spectral information and serves as an excitation source for a widefield imaging system. (*Middle*) Pseudo-colored signal (fire) in the NIR channel of the widefield imaging system and the spectroscopic signal from within the contrast-enhanced region; (*Bottom*) NIR signal overlaid (cyan) onto the color image shows robust enhancement of syngeneic, orthotopic pancreatic cancer. Contrast enhancement can be observed with HA<sub>5k</sub>-, HA<sub>20k</sub>- and HA<sub>100k</sub>-Cy7.5, HA<sub>5k</sub>- and HA<sub>100k</sub>-IRDye800 (see inset middle row).



**Figure S6.** *Ex vivo* analysis of HA<sub>20k</sub>-dye accumulation in healthy pancreas from W.T. C57BL/6 mice 24 h post injection. (*Top row*) NIRF channel and white field channel merged image of a representative pancreas marked with acquisition locations of the spectroscopic semi-quantification shown at the in *middle row*, fluorescence intensity excited with medium laser power and 1 s integration by the spectroscopic pen component of FIGS system; (*Bottom row*) plots of intensity

values along the red dashed line from the NIRF images quantified by ImageJ;



Figure S7. *Ex vivo* analysis of HA-dye accumulation in pancreatic tumor (*left column* for each group) or healthy pancreas from W.T. C57BL/6 mice (*right column* for each group) 96 h post

injection. (*Top row*) NIRF channel and white field channel merged image of a representative pancreas marked with acquisition locations of the spectroscopic semi-quantification shown at the in *middle row*, fluorescence intensity excited with medium laser power and 1 s integration by the spectroscopic pen component of FIGS system; (*Bottom row*) plots of intensity values along the red dashed line from the NIRF images quantified by ImageJ; (I) contrast within PDAC-bearing pancreas as determined by muscle SNR weighted SNR of PDAC, uninvolved pancreas (Uni panc.). and healthy pancreas (Hpanc) from WT C57BL/6. Data were collected 4 day post *i.v.* injection of contrast agents by tail vein. SNR value were calculated based of fluorescence intensity in NIRF images. \*\*\*p < 0.001, \*\*p < 0.01, \*p < 0.05. N = 4-5 for each group.



**Figure S8**. CD44 expression in PDAC cells *ex vivo* and *in vitro*. (A) Representative flow cytometry analytical histogram of untreated, primary pancreatic epithelial (PPE) and KPC cells single stained with CD44-FITC; (B) histology of PDAC-bearing murine pancreas; (a) scanned H&E stained murine PDAC; (b) mouse PDAC with 100 × magnification; (c) scanned CD44 stained slides of murine PDAC; (d) microscopic capture of mouse PDAC with 100 × magnification; (e) microscopic capture of mouse sarcomatoid with 100 × magnification. Scale bar represents 2 mm for scanned images and 10 µm in captured microscopic capture; (C) schematic representation of HA-dye bind to CD44 receptors, which were expressed intensively on pancreatic cancer cell but moderately on normal pancreatic cells.

Acute toxicity of HA<sub>20k</sub>-NIRF was examined to further confirm their translational potential (Figure S9-10). Upon the administration of HA<sub>20k</sub>-NIRF conjugates at 20 times of the dose used for imaging, a comprehensive diagnostic profile was determined for indications of toxicities in Disorders including malnutrition, liver. kidney, bone, GI, pancreas. dehydration, hyperglycemia/hypoglycemia were assessed. A slight increase of BUN for HA<sub>20k</sub>-IRDye800 could be attributed to its renal clearance (Figure S9). Whereas reduced amount of total protein from HA<sub>20k</sub>-Cy7.5 might be correlated with the strong protein adsorption (Figure S9). However, the lungs, kidneys, spleen, and heart were histologically unremarkable by H&E staining (Figure S10). Liver sections from each animal demonstrated mild, diffuse microvesicular steatosis within hepatocytes in the absence of lobular/portal inflammation, ballooning degeneration, or apoptosis. Brain sections overall were without abnormality with occasional animals showing scattered red neurons in Purkinje cells within the cerebellum.



**Figure S9.** Comprehensive biochemical and hematological assessments for WT C57BL/6 mice injected with HA<sub>20k</sub>-NIRF (equivalent to 20 nmol of free dye/mouse) or vehicle control for the acute toxicology study. N = 3. ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AMY, amylase; BUN, blood urea nitrogen; CA, calcium; PHOS, phosphorus;

CRE, Creatinine; GLU, glucose; TP, total protein; GLOB, globulin; WBC, white blood cells; RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit.



**Figure S10.** Representative images for the histological assessments of vital organs harvested from WT C57BL/6 mice injected with HA<sub>20k</sub>-NIRF (equivalent to 20 nmol of free dye/mouse) or vehicle control. N = 3.

	HA <sub>5k</sub> - Cy7.5	HA <sub>20k</sub> - Cy7.5	HA <sub>100k</sub> - Cy7.5	HA <sub>5k</sub> - IRDye800	HA <sub>20k</sub> - IRDye800	HA <sub>100k</sub> - IRDye800
Weight ratio (g/g) dye/polymer	0.57	0.15	0.10	0.26	0.05	0.02
Dyes per chain	1.23	1.15	0.56	1.18	0.69	1.40
Conjugating efficiency (%)	77.1	88.8	78.9	57.8	27.8	18.4
Yield (%)	37.3	33.3	21.8	45.6	68.5	75.5

Table S1. Chemical Characteristics of HA-dye conjugates

Table S2. Ratio of HA-Cy7.5-treated pancreas or PDAC fluorescence divided by signal of surrounding organs 24 h post injection (N = 5)

	W.T. C57BL/6				РДАС			
	Cy7.5	HA <sub>5k</sub> -Cy7.5	HA <sub>20k</sub> -Cy7.5	HA <sub>100k</sub> - Cy7.5	Cy7.5	HA <sub>5k</sub> -Cy7.5	HA <sub>20k</sub> -Cy7.5	HA <sub>100k</sub> - Cy7.5
Muscle	2.31±0.50	4.64±1.29	4.46±1.21	4.44±1.27	3.59±1.68	27.56±4.83	33.88±11.98	40.76±20.90
Stomach	0.78±0.31	$0.58 \pm 0.08$	0.60±0.36	0.79±0.12	0.36±0.11	4.98±1.78	5.45±1.28	6.94±2.10
Int	2.38±0.41	$0.42 \pm 0.20$	0.41±0.07	0.62±0.39	1.77±0.75	3.67±1.19	5.77±1.50	10.06±3.09
Liver	0.04±0.01	0.02±0.003	0.02±0.01	0.03±0.01	0.05±0.02	0.25±0.06	0.51±0.11	0.43±0.25
Kidney	0.08±0.01	0.11±0.02	0.29±0.12	0.12±0.04	0.12±0.04	2.02±0.42	4.68±0.28	6.93±2.74
Spleen	0.56±0.17	0.14±0.03	0.12±0.08	0.24±0.12	0.69±0.40	1.18±0.41	1.76±0.32	1.11±0.40

Table S3. Ratio of HA-IRDye800-treated pancreas or PDAC fluorescence divided by signal of surrounding organs 24 h post injection (N = 5)

	W.T. C57BL/6				PDAC			
	IRDye	HA <sub>5k</sub> - IRDye	HA <sub>20k</sub> - IRDye	HA <sub>100k</sub> - IRDye	IRDye	HA <sub>5k</sub> -IRDye	HA <sub>20k</sub> -IRDye	HA <sub>100k</sub> - IRDye
Muscle	3.38±0.85	0.92±0.78	2.26±0.38	1.74±0.59	8.28±5.55	9.96±5.63	29.81±11.26	12.42±9.00
Stomach	0.17±0.03	0.39±0.58	0.23±0.08	0.17±0.05	1.06±0.59	1.54±0.97	3.50±1.32	1.04±0.77
Int	1.47±0.70	0.54±0.50	1.44±0.20	1.14±0.34	6.00±3.17	11.03±5.90	25.56±10.74	8.59±7.16
Liver	0.08±0.03	0.11±0.09	0.09±0.01	0.04±0.01	0.36±0.14	1.65±0.66	1.81±0.69	0.30±0.20
Kidney	0.12±0.02	0.10±0.08	0.19±0.03	0.22±0.06	0.52±0.22	1.14±0.48	1.96±0.34	1.06±0.65
Spleen	0.59±0.10	0.43±0.26	0.51±0.06	0.20±0.10	2.08±1.14	5.01±1.73	3.60±0.72	0.91±0.58

Abbreviations used are: Int: small intestines