Supporting Information

Multicolor Fluorescence Imaging of Traumatic Brain Injury in a Cryolesion Mouse Model

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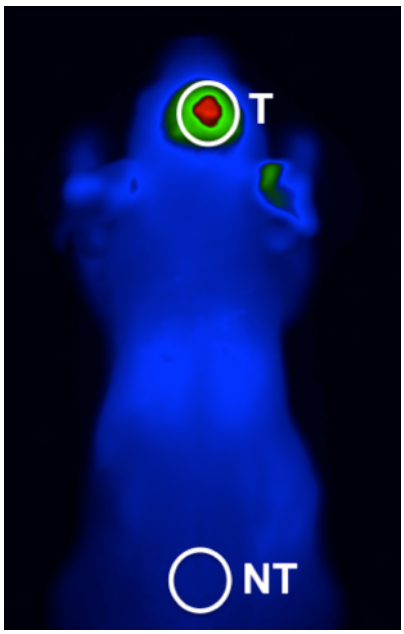

Figure S1. Representative *in vivo* epi-flluorescence image with typical region of interest (ROI) shapes used to measure mean pixel intensities for the target (T) and the non-target (NT).

Table S1. *In vivo* T/NT values for PSS-794, Tracer-794, and Annexin-Vivo 750 The data is displayed in graphical form in Figure 3. T/NT values ± standard error of the mean.

	PSS-794	**Tracer-794**	**Annexin-Vivo 750**
0 h	2.52 ± 0.07	2.52 ± 0.42	2.57 ± 0.24
3 h	6.77 ± 0.47***	3.52 ± 0.59	3.69 ± 0.49***
6 h	7.32 ± 0.78*	3.81 ± 0.90	4.09 ± 0.28*
24 h	5.93 ± 1.15	2.97 ± 0.69	4.87 ± 0.54

* $P < 0.01$
** $P < 0.02$
*** $P < 0.003$

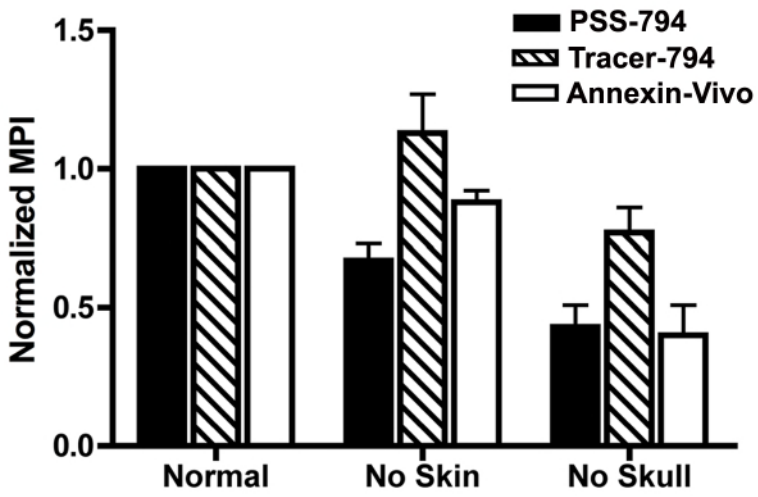

Figure S2. Comparison of probe localization at the cryoinjury site in different tissues. A pre-cooled metal cylinder was applied to the heads of mice for 60 s, and each mouse was dosed with either PSS-794, Tracer-794, or Annexin-Vivo 750. The mice were imaged 24 h later, sacrificed and imaged again with the skin removed (no skin), and with both the skin and skull removed (no skull). With each image, a region of interest (ROI) was drawn around the cryoinjury site; the mean pixel intensity (MPI) was recorded and normalized to the mean pixel intensity of the *in vivo* (normal) image. Normalized MPI ± standard error of the mean. N = 5.

Table S2. Normalized mean pixel intensities of PSS-794, Tracer-794, and Annexin-Vivo 750 at the cryoinjury site. The data is displayed in graphical form in Figure S2. Normalized mean pixel intensities ± standard error of the mean.

	PSS-794	**Tracer-794**	**Annexin-Vivo 750**
Normal	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
No Skin	0.67 ± 0.06^{*a}	1.14 ± 0.14	0.88 ± 0.04^{b}
No Skull	0.43 ± 0.08^{**a}	0.77 ± 0.09	0.40 ± 0.11^{b}

^{a}PSS-794 No Skin vs No Skull = $P < 0.02$

^{b}Annexin-Vivo No Skin vs No Skull = $P < 0.03$

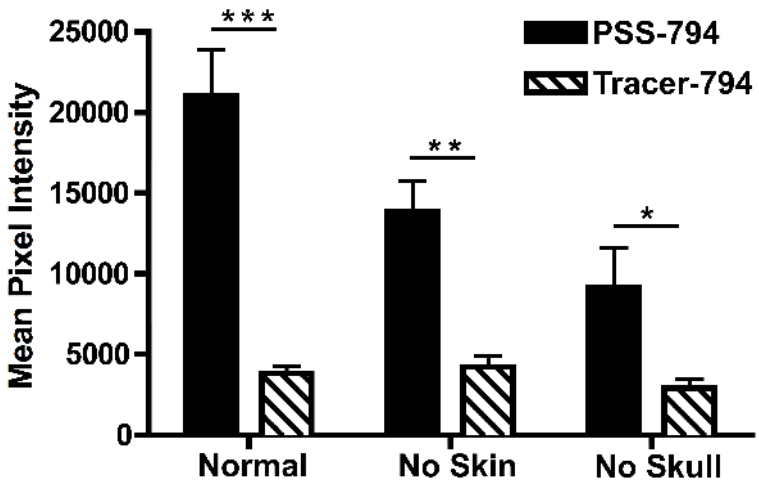

Figure S3. Absolute mean pixel intensities used to create the data in Figure S2 and Table S2. Error bars are ± standard error of the mean. ***** $P < 0.03$, ****** $P < 0.001$, ******* $P < 0.0005$. $N = 5$.

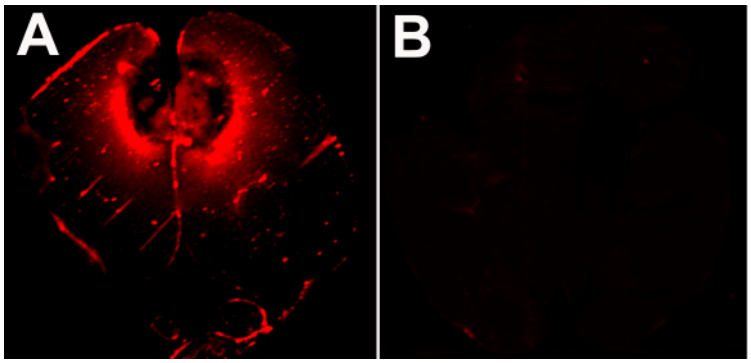

Figure S4. *Ex vivo* near-infrared fluorescence images of cryoinjured brain sections containing PSS-794 (A) or Tracer-794 (B). A pre-cooled metal cylinder was applied to the heads of mice for 60 s, and each mouse was dosed with either PSS-794 (3.0 mg/kg) or Tracer-794 (3.0 mg/kg). After an additional 24 h, the mice were sacrificed and the brains were excised. Brains were flash frozen in OCT and cryosectioned at 10 μM thickness. The images show PSS-794 around the site of cryolesion, while a negligible amount of Tracer-794 remains in the brain after 24 h.

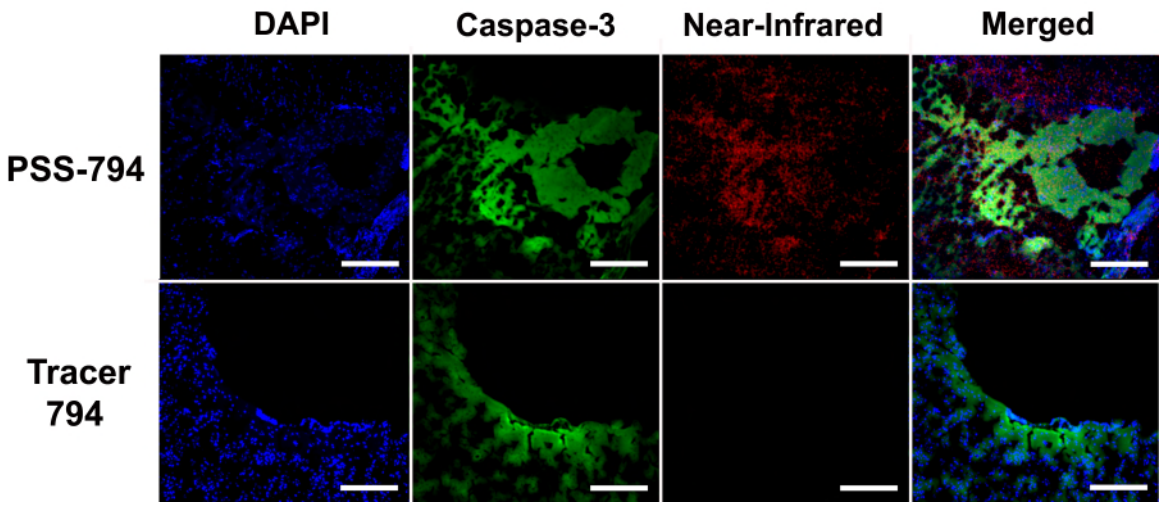

Figure S5. Fluorescence microscopy of brain cryoinjury histological sections. Mice received a 60 s brain cryoinjury and were immediately injected with either PSS-794 or Tracer-794. Mice were sacrificed 24 h post-probe injection and the brains were excised and flash frozen in OCT. The brains were cut into 10 μm sections and counterstained with DAPI and anti-caspase-3 antibody. Images were acquired at the site of the cryoinjury. Scale bar = 200 μm.

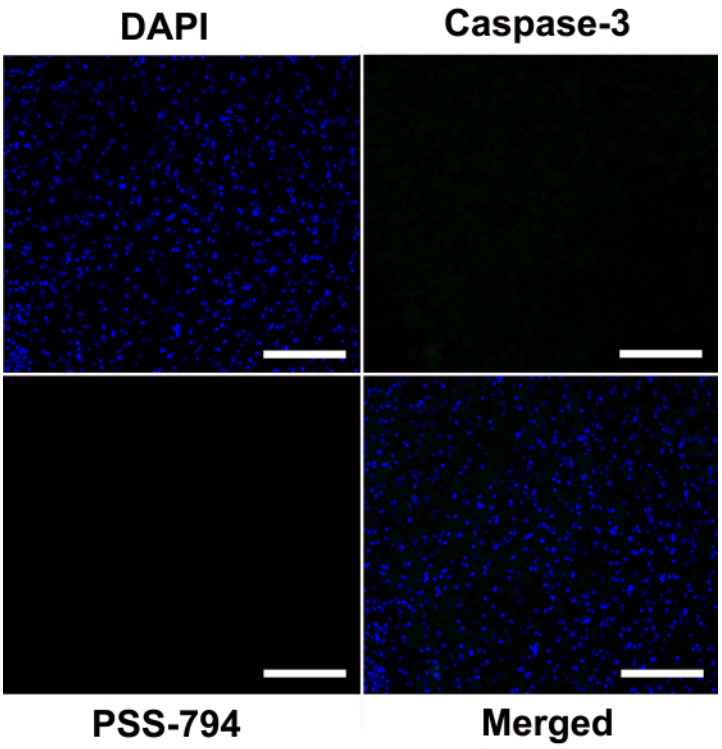

Figure S6. Fluorescence microscopy of histological sections from healthy brain tissue. Mice received a 60 s brain cryoinjury and were immediately injected with PSS-794. Mice were sacrificed 24 h post-probe injection and the brains were excised and flash frozen in OCT. The brains were cut into 10 μm sections and counterstained with DAPI and anti-caspase-3 antibody. Images were acquired in an area of the brain that was not affected by the cryoinjury. Scale bar = 200 μm.

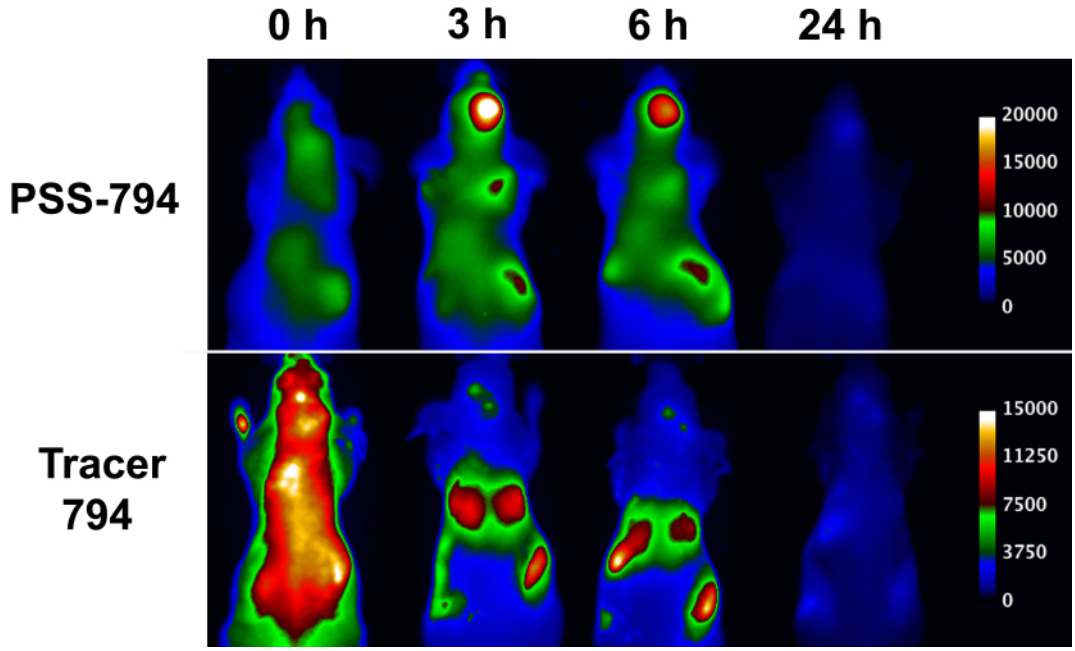

Figure S7. Representative *in vivo* near-infrared fluorescence montage of mice with a 20 s brain cryoinjury. Both cohorts of mice were anesthetized and a pre-cooled metal cylinder was applied to each mouse's head for 20 s. The mice were dosed with either PSS-794 or Tracer-794 immediately after the cryoinjury. Images were acquired at the indicated time points after probe injection. The calibration bar applies to all images. N = 5.

Table S3. T/NT values for PSS-794 and Tracer-794 at the cryolesion site in the 60 s and 20 s brain cryolesion mouse model. The data is displayed in graphical form in Figure 5. T/NT values ± standard error of the mean.

	PSS-794		**Tracer-794**	
	60 s cryolesion	20 s cryolesion	60 s cryolesion	20 s cryolesion
0 h	2.52 ± 0.07	2.48 ± 0.12	2.52 ± 0.42	2.00 ± 0.16
3 h	6.77 ± 0.47§§§	4.67 ± 0.24**	3.52 ± 0.59	2.36 ± 0.31
6 h	7.32 ± 0.78§	4.07 ± 0.72*	3.81 ± 0.90	2.45 ± 0.41
24 h	5.93 ± 1.15§§	2.68 ± 0.20	2.97 ± 0.69	2.38 ± 0.35

* $P < 0.03$, ** $P < 0.002$

§ $P < 0.05$, §§ $P < 0.02$, §§§ $P < 0.01$ (when comparing 60 s to 20 s cryolesion)

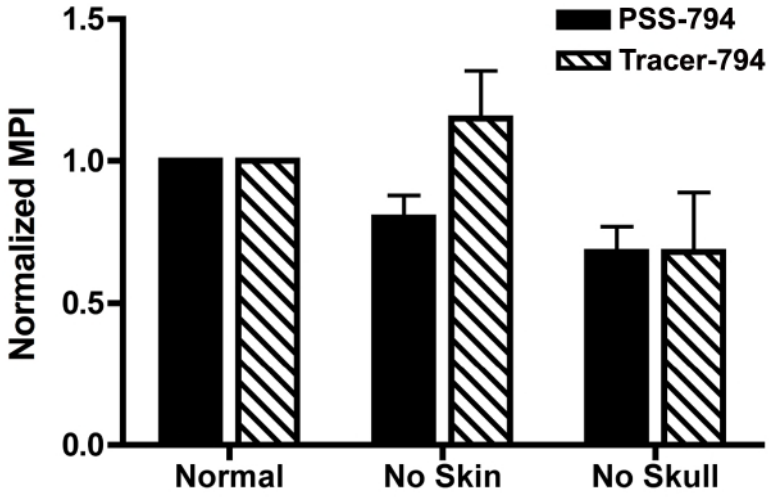

Figure S8. Comparison of PSS-794 and Tracer-794 localization at the cryoinjury site in different tissues. A pre-cooled metal cylinder was applied to the heads of mice for 20 s, and each mouse was dosed with either PSS-794 or Tracer-794. A region of interest (ROI) was drawn around the cryoinjury site in the 24 h *in vivo* (normal) images, images with the skin removed (no skin), and images with both the skin and skull removed (no skull) at 24 h post-probe injection. The mean pixel intensities (MPI) were recorded and normalized to the mean pixel intensity from the *in vivo* image. Normalized MPI ± standard error of the mean. N = 5.

Table S4. Normalized mean pixel intensities of PSS-794 and Tracer-794 at the cryoinjury site in the 20 s brain cryoinjury mouse model. The data is displayed in graphical form in Figure S8. Normalized mean pixel intensities ± standard error of the mean.

	PSS-794	**Tracer-794**
Normal	1.00 ± 0.00	1.00 ± 0.00
No Skin	0.80 ± 0.08	1.15 ± 0.17
No Skull	0.68 ± 0.09	0.68 ± 0.21

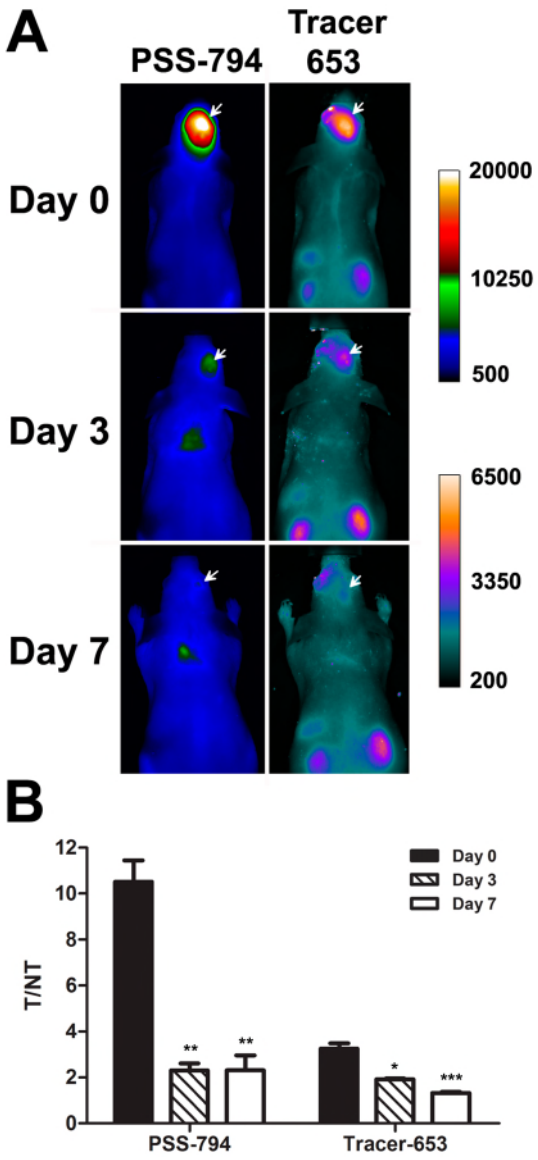

Figure S9. Representative *in vivo* near-infrared fluorescence montages of PSS-794 and Tracer-653 accumulation in a brain cryoinjury mouse model (A). Three cohorts of hairless mice were given a 60 s brain cryoinjury. Mice were then injected with a single dose of PSS-794 either immediately following cryoinjury (Day 0), 3 days post-cryoinjury (Day 3), or 7 days post-cryoinjury (Day 7). Each mouse was also injected with Tracer-653 at five hours post-PSS-794 injection. One hour after Tracer-653 injection, the mice were anesthetized and subjected to non-invasive epi-fluorescence imaging. Arrows denote the location of the cryolesion. *In vivo* quantification of PSS-794, and Tracer-653 accumulating in a 60 s brain cryoinjury mouse model (B). Target to non-target ratios (T/NT) were calculated by region of interest (ROI) analysis of the digital images. *P < 0.005, **P < 0.001. ***P < 0.0002.

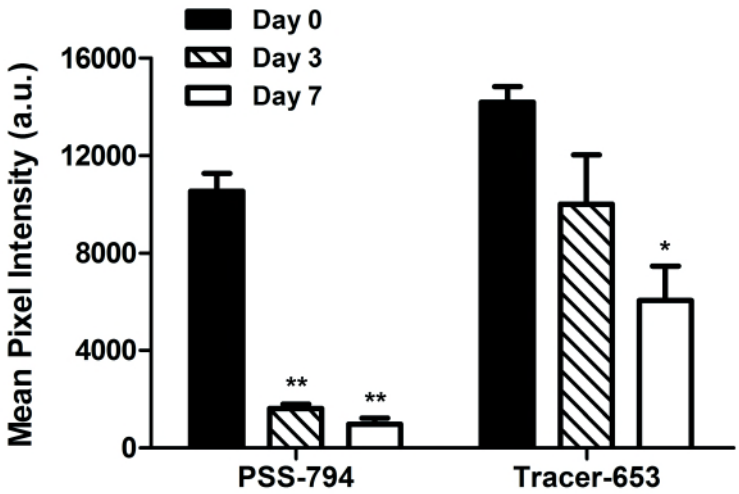

Figure S10. Comparison of PSS-794 and Tracer-653 in brain sections taken from mice that were dosed with probe at the following timepoints after cryoinjury, Day 0, Day 3, and Day 7. A region of interest was drawn around the area of the cryoinjury on the brain, and the mean pixel intensity was recorded. Mean pixel intensity ± standard error of the mean. *P < 0.05, **P < 0.0002. N = 3-4.

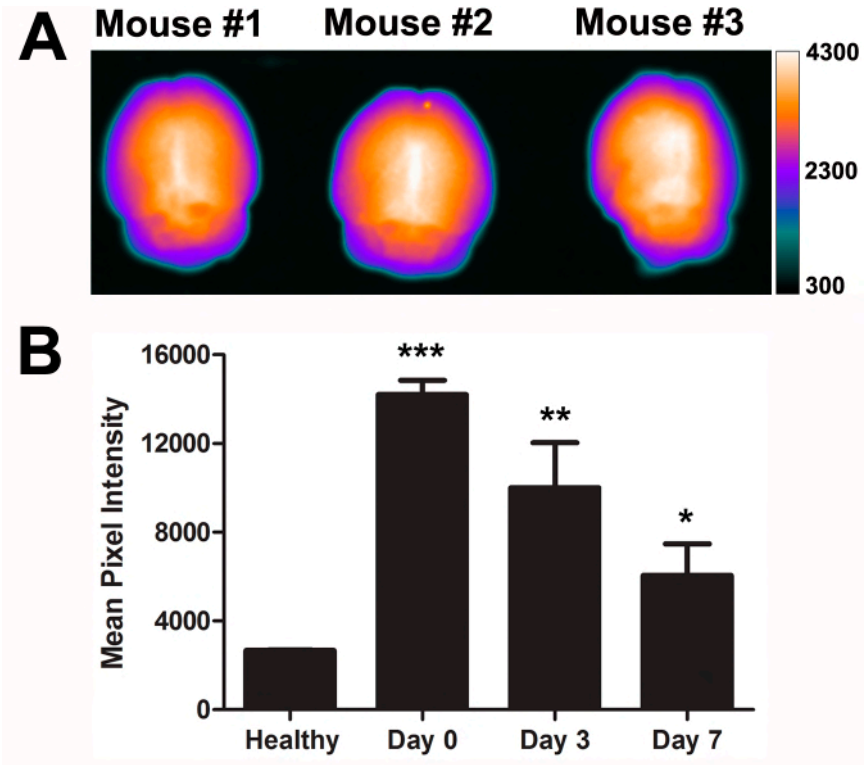

Figure S11. Comparison of Tracer-653 accumulation in healthy mouse brains and in cryoinjured mouse brains. *Ex vivo* fluorescence images of brains from healthy, hairless mice that were injected with Tracer-653 (A). Healthy, hairless mice (n = 3) were injected intraveneously with Tracer-653 (2 mg/kg in 100 μL H_2O). Following one hour, the mice were anesthetized, sacrificed, and the brains were excised for *ex vivo* epi-fluorescence imaging. Calibration bar applies to all images. Bar graph of Tracer-653 mean pixel intensities of brains taken from healthy mice or cryoinjured mice that were dosed with probe at the designated timepoints after cryoinjury, (B). A region of interest was drawn around the area of the cryolesion on the injured brain or on a similar anatomical location on the healthy brain, and the mean pixel intensity was recorded. Mean pixel intensity ± standard error of the mean. *P < 0.05, **P < 0.02, ***P < 0.0001 when compared to healthy. N = 3-4.

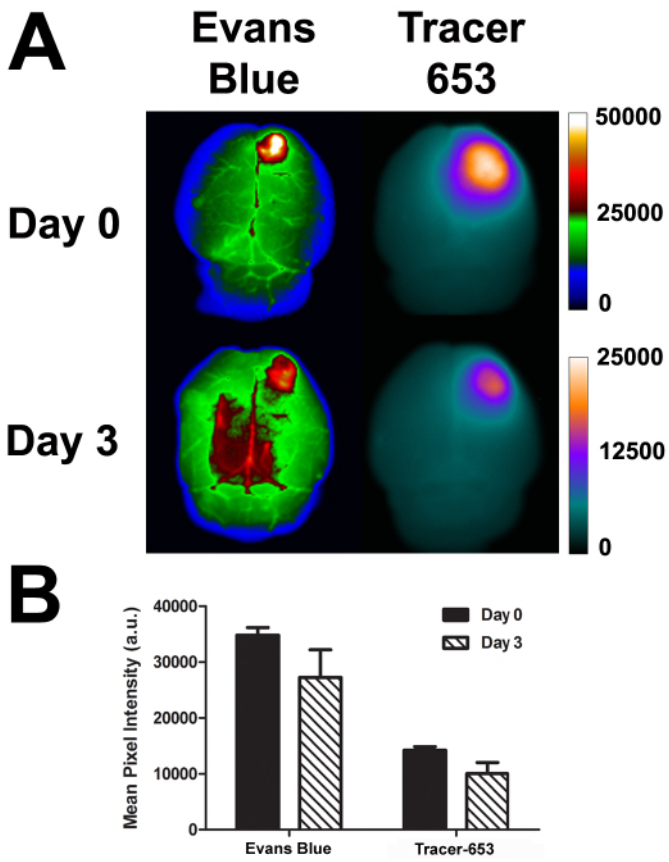

Figure S12. Comparison between Evans Blue and Tracer-653 accumulation into cryoinjured mouse brains. *Ex vivo* fluorescence images of Evans Blue and Tracer-653 in brains excised from mice that were dosed with probe either 6 h (Day 0) or 3 days (Day 3) after cryoinjury (A). Bar graph of Evans Blue and Tracer-653 mean pixel intensities at the site of the cryoinjury (B). A region of interest was drawn around the area of the cryoinjury on the brain, and the mean pixel intensity was recorded. Mean pixel intensity ± standard error of the mean. N = 3-4.