

## SUPPLEMENTARY INFORMATION: 'In Situ Excitation of BODIPY fluorophores by $^{89}\text{Zr}$ -generated Cerenkov luminescence'

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### Supplementary Fig. 1 – Spectro-fluorophotometer BODIPY emission spectra

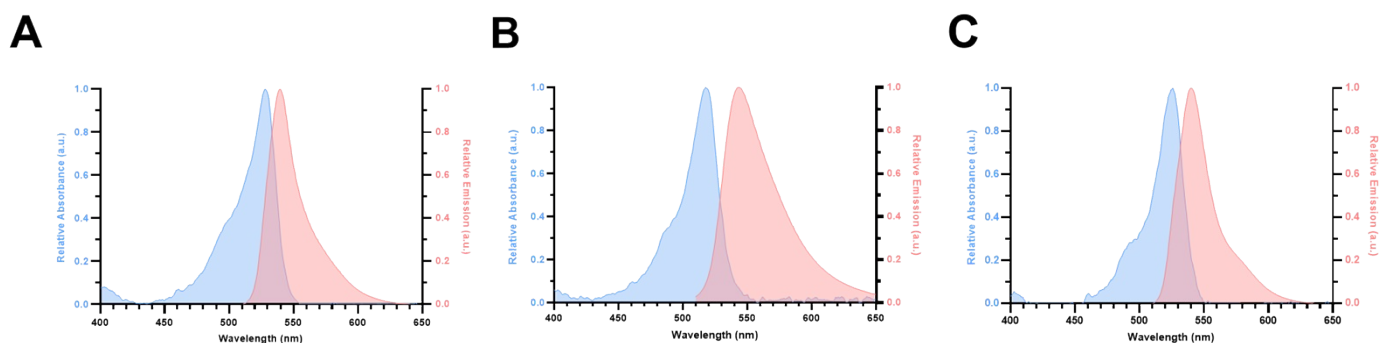


Fig. S1 Relative BOD-H, BOD-Me and BOD-Ph absorption and emission spectra in DMSO. Absorbance (blue) data collected on Nanodrop One C for each BODIPY at the following concentrations: 5  $\mu\text{M}$  (BOD-H), 10  $\mu\text{M}$  (BOD-Me), 2.5  $\mu\text{M}$  (BOD-Ph). Emission data (red dots) collected on RF-6000 spectro-fluorophotometer (Shimadzu) at the same concentrations. A) BOD-H (*{3-Ethyl-5-[(4-ethyl-3,5-dimethyl-2H-pyrrol-2-ylidene)methyl]-2,4-dimethyl-1H-pyrrolato-N1,N5}difluoroboron*); excitation wavelength = 528 nm, emission maximum = 540 nm. B) BOD-Me (*{Difluoro[3-ethyl-5-[1-(4-ethyl-3,5-dimethyl-2H-pyrrol-2-ylidene-N)ethyl]-2,4-dimethyl-1H-pyrrolato-N]boron*); excitation wavelength = 518 nm, emission maximum = 538 nm. C) BOD-Ph (*{2,8-Diethyl-1,3,5,7-tetramethyl-9-phenylbipyromethene difluoroborate}*); excitation wavelength = 526 nm, emission maximum = 540 nm. Analysed on GraphPad Prism (version 9.2.0 for Windows, GraphPad Software).

### Supplementary Fig. 2 – $^{89}\text{Zr}$ half-life analysis

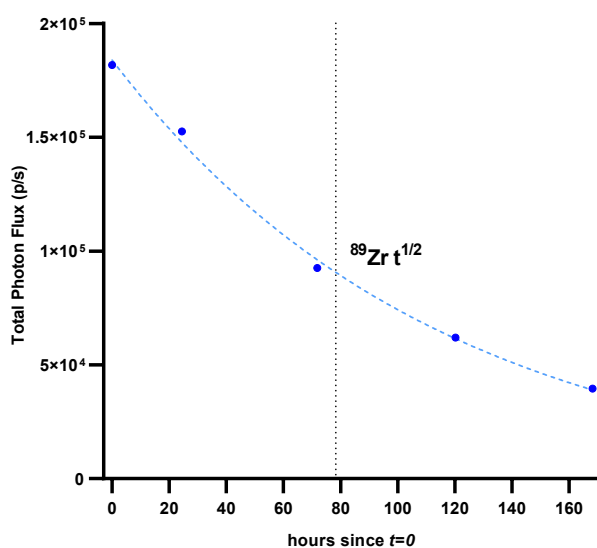


Fig. S2.  $^{89}\text{Zr}$  decay analysis. One-phase decay analysis performed on 0.25 MBq  $^{89}\text{Zr}$  in DMSO (2.5 kBq/ $\mu\text{l}$ ). Cerenkov luminescence total photon flux (photons/second; p/s) measured on IVIS Spectrum over 168 hours (blue points) and one-phase decay model (blue dashed line) fit using GraphPad Prism (version 9.2.0 for Windows, GraphPad Software).  $R^2 = 0.98$ , experimental half-life = 78.3 h  $\pm$  6.9 h (SD) (black dashed line) compared to known  $^{89}\text{Zr}$  half-life of 78.41 h.

Supplementary Table 1 – Full range of photon flux decay data for BOD-H

Ratio <sup>89</sup> Zr (kBq):[BODIPY] (μM)	Time (h)	Average Photon Flux (p/s) ± SD (p/s)
0.25:1	0	4.80E+05 ± 3.89E+05
	24	3.68E+05 ± 3.29E+05
	72	2.46E+05 ± 2.13E+05
	120	1.67E+05 ± 1.35E+05
	168	1.05E+05 ± 8.93E+04
0.38:1	0	2.07E+05 ± 1.38E+04
	24	1.61E+05 ± 1.84E+04
	72	1.02E+05 ± 9.83E+03
	120	7.84E+04 ± 1.25E+04
	168	5.04E+04 ± 5.06E+03
0.58:1	0	1.81E+05 ± 6.26E+04
	24	1.41E+05 ± 3.45E+04
	72	9.42E+04 ± 2.47E+04
	120	5.51E+04 ± 1.63E+04
	168	3.84E+04 ± 8.62E+03
0.88:1	0	1.46E+05 ± 5.22E+04
	24	1.13E+05 ± 5.32E+04
	72	8.23E+04 ± 2.56E+04
	120	5.51E+04 ± 1.63E+04
	168	3.25E+04 ± 9.82E+03
1.34:1	0	1.18E+05 ± 3.99E+04
	24	9.10E+04 ± 3.26E+04
	72	6.76E+04 ± 2.08E+04
	120	3.99E+04 ± 8.48E+03
	168	2.62E+04 ± 6.00E+03
2.03:1	0	1.04E+05 ± 4.69E+04
	24	7.90E+04 ± 4.74E+04
	72	4.50E+04 ± 2.62E+04
	120	3.48E+04 ± 9.20E+03
	168	1.90E+04 ± 4.91E+03
3.09:1	0	8.88E+04 ± 3.65E+04
	24	5.82E+04 ± 3.28E+04
	72	4.51E+04 ± 1.98E+04
	120	2.26E+04 ± 1.07E+04
	168	1.88E+04 ± 7.23E+03
4.72:1	0	5.72E+04 ± 2.62E+04
	24	4.76E+04 ± 2.80E+04
	72	2.80E+04 ± 1.50E+04
	120	2.28E+04 ± 9.39E+03
	168	9.25E+03 ± 3.63E+03
7.14:1	0	3.54E+04 ± 2.99E+04
	24	2.56E+04 ± 2.06E+04
	72	1.40E+04 ± 9.52E+03
	120	1.48E+04 ± 9.48E+03
	168	8.57E+03 ± 7.43E+03
10.87:1	0	4.59E+04 ± 3.41E+04
	24	2.30E+04 ± 1.40E+04
	72	1.58E+04 ± 1.13E+04
	120	1.35E+04 ± 6.31E+03
	168	7.01E+03 ± 1.59E+03
16.67:1	0	3.31E+04 ± 2.80E+04
	24	1.70E+04 ± 1.58E+04
	72	1.73E+04 ± 8.02E+03
	120	2.43E+03 ± 6.35E+03
	168	3.32E+03 ± 6.63E+03
25.00:1	0	3.34E+04 ± 1.95E+04
	24	9.82E+03 ± 1.48E+04
	72	5.91E+03 ± 1.36E+04
	120	1.12E+03 ± 5.02E+03
	168	3.55E+03 ± 1.00E+04

Supplementary Table 2 – Full range of photon flux decay data for BOD-Me

Ratio <sup>89</sup> Zr (kBq):[BODIPY] (μM)	Time (h)	Average Photon Flux (p/s) ± SD (p/s)
0.25:1	0	3.25E+05 ± 1.80E+04
	24	2.54E+05 ± 2.17E+04
	72	1.74E+05 ± 1.76E+04
	120	9.75E+04 ± 2.46E+04
	168	3.35E+04 ± 1.54E+04
0.38:1	0	2.92E+05 ± 4.07E+04
	24	2.29E+05 ± 1.42E+04
	72	1.60E+05 ± 1.11E+04
	120	1.01E+05 ± 1.10E+04
	168	6.60E+04 ± 6.53E+03
0.58:1	0	2.38E+05 ± 3.03E+04
	24	1.76E+05 ± 1.15E+04
	72	1.24E+05 ± 6.68E+03
	120	7.76E+04 ± 1.28E+04
	168	4.56E+04 ± 3.15E+03
0.88:1	0	2.03E+05 ± 3.88E+04
	24	1.55E+05 ± 2.73E+04
	72	9.57E+04 ± 1.88E+04
	120	6.19E+04 ± 1.52E+04
	168	4.07E+04 ± 8.56E+03
1.34:1	0	1.53E+05 ± 9.10E+03
	24	1.03E+05 ± 2.08E+04
	72	7.08E+04 ± 1.89E+04
	120	4.16E+04 ± 1.04E+04
	168	2.81E+04 ± 9.52E+03
2.03:1	0	1.28E+05 ± 3.95E+04
	24	8.87E+04 ± 1.97E+04
	72	5.80E+04 ± 1.44E+03
	120	3.79E+04 ± 4.30E+03
	168	2.31E+04 ± 6.03E+03
3.09:1	0	9.83E+04 ± 4.44E+04
	24	6.82E+04 ± 1.77E+04
	72	4.18E+04 ± 1.73E+04
	120	2.33E+04 ± 1.08E+04
	168	1.26E+04 ± 5.03E+03
4.72:1	0	7.66E+04 ± 2.40E+04
	24	4.15E+04 ± 1.68E+04
	72	4.44E+04 ± 6.83E+03
	120	2.44E+04 ± 1.46E+03
	168	1.35E+04 ± 3.70E+03
7.14:1	0	6.92E+04 ± 2.85E+04
	24	3.91E+04 ± 1.42E+04
	72	3.02E+04 ± 2.04E+03
	120	1.85E+04 ± 7.33E+03
	168	1.18E+04 ± 4.26E+03
10.87:1	0	5.60E+04 ± 1.62E+04
	24	2.38E+04 ± 1.92E+04
	72	1.78E+04 ± 1.37E+04
	120	7.28E+03 ± 5.27E+03
	168	5.86E+03 ± 6.37E+03
16.67:1	0	3.44E+04 ± 2.99E+04
	24	1.20E+04 ± 1.88E+04
	72	1.22E+04 ± 1.48E+04
	120	7.54E+02 ± 1.39E+04
	168	-1.53E+03 ± 6.86E+03
25.00:1	0	1.87E+04 ± 1.35E+04
	24	-2.01E+03 ± 1.50E+04
	72	2.60E+03 ± 1.68E+04
	120	-1.20E+04 ± 2.08E+04
	168	-3.72E+03 ± 6.62E+03

Supplementary Table 3 – Full range of photon flux decay data for BOD-Ph

Ratio <sup>89</sup> Zr (kBq):[BODIPY] (μM)	Time (h)	Average Photon Flux (p/s) ± SD (p/s)
0.25 : 1	0	2.73E+05 ± 4.45E+04
	24	2.00E+05 ± 2.36E+04
	72	1.27E+05 ± 1.25E+04
	120	8.07E+04 ± 8.16E+04
	168	5.03E+04 ± 6.58E+03
0.38 : 1	0	2.47E+05 ± 6.68E+04
	24	1.94E+05 ± 5.04E+04
	72	1.19E+05 ± 3.52E+04
	120	7.59E+04 ± 2.63E+04
	168	4.67E+04 ± 1.29E+04
0.58 : 1	0	2.07E+05 ± 4.51E+04
	24	1.59E+05 ± 4.78E+04
	72	9.85E+04 ± 2.14E+04
	120	6.71E+04 ± 1.48E+04
	168	4.10E+04 ± 1.08E+04
0.88 : 1	0	1.61E+05 ± 4.75E+04
	24	1.30E+05 ± 4.41E+04
	72	8.43E+04 ± 2.00E+04
	120	5.58E+04 ± 8.82E+03
	168	3.57E+04 ± 1.45E+04
1.34 : 1	0	1.43E+05 ± 4.44E+04
	24	1.10E+05 ± 3.72E+04
	72	6.87E+04 ± 1.98E+04
	120	4.92E+04 ± 1.09E+04
	168	2.80E+04 ± 8.53E+03
2.03 : 1	0	8.16E+04 ± 8.49E+03
	24	6.40E+04 ± 2.03E+04
	72	4.61E+04 ± 9.94E+03
	120	2.82E+04 ± 1.27E+04
	168	1.73E+04 ± 4.83E+03
3.09 : 1	0	7.53E+04 ± 3.36E+04
	24	6.21E+04 ± 2.03E+04
	72	4.31E+04 ± 9.96E+03
	120	2.60E+04 ± 7.61E+03
	168	1.49E+04 ± 1.09E+04
4.72 : 1	0	4.07E+04 ± 3.53E+04
	24	4.18E+04 ± 4.17E+04
	72	3.03E+04 ± 1.64E+04
	120	1.61E+04 ± 9.86E+03
	168	1.08E+04 ± 1.07E+04
7.14 : 1	0	4.76E+04 ± 2.70E+04
	24	3.92E+04 ± 3.16E+04
	72	3.20E+04 ± 1.49E+04
	120	1.75E+04 ± 9.07E+03
	168	9.89E+03 ± 7.52E+03
10.87 : 1	0	1.62E+04 ± 6.15E+03
	24	2.65E+04 ± 2.54E+04
	72	2.56E+04 ± 7.89E+03
	120	1.13E+04 ± 1.19E+04
	168	7.62E+03 ± 5.15E+03
16.67 : 1	0	2.73E+04 ± 2.22E+04
	24	2.68E+04 ± 2.18E+04
	72	2.16E+04 ± 1.02E+04
	120	1.15E+04 ± 4.52E+03
	168	6.95E+03 ± 4.16E+03
25.00 : 1	0	1.08E+04 ± 2.01E+04
	24	2.08E+04 ± 1.77E+04
	72	1.24E+04 ± 1.92E+04
	120	6.42E+03 ± 9.99E+03
	168	2.03E+03 ± 7.45E+03

### Supplementary Fig. 3 – Light-excitation of BODIPY serial dilutions

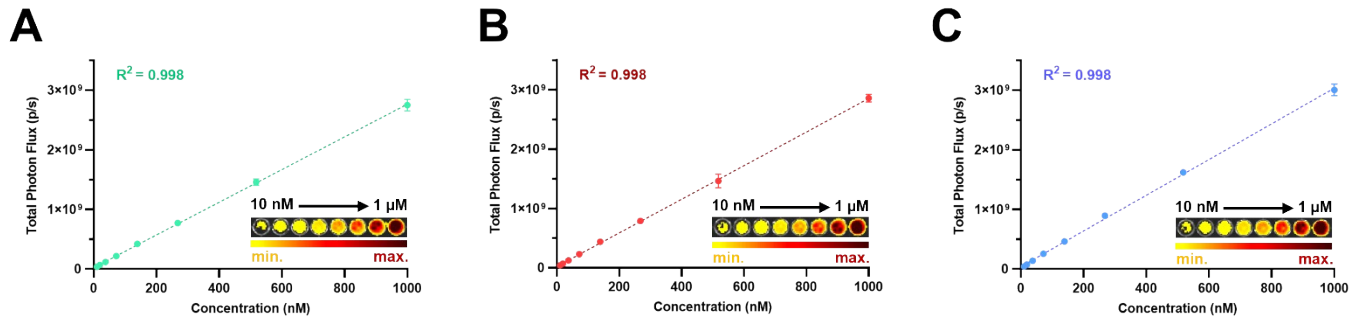


Fig. S3. Fluorescence intensity against BODIPY concentration following light excitation; A BOD-H, B BOD-Me, C BOD-Ph. Serial dilutions (1  $\mu$ M to 10 nM; DF = 1.9307) of each BODIPY dye (in DMSO) were performed in triplicate. Concentration range is lower than the range used to investigate SCIFI intensity against BODIPY concentration (Fig. 4) to avoid saturation of the light detector in the imaging system, as conventional light excitation produces significantly greater fluorescence intensity compared to Cerenkov luminescence, reflected in the y-axis of the figures. Total photon flux (p/s) [dots] measurements were taken on an *in vitro* imaging system using a 540 nm emission filter following excitation with a 500 nm light source with an exposure time of 1 second. Linear regression analysis of the data was performed [dashed line &  $R^2$  value] and revealed no quenching of the dyes at higher concentrations. IVIS images of the wells are shown in inset panels and are normalised to the maximum photon flux (p/s) value. Error bars = SD.

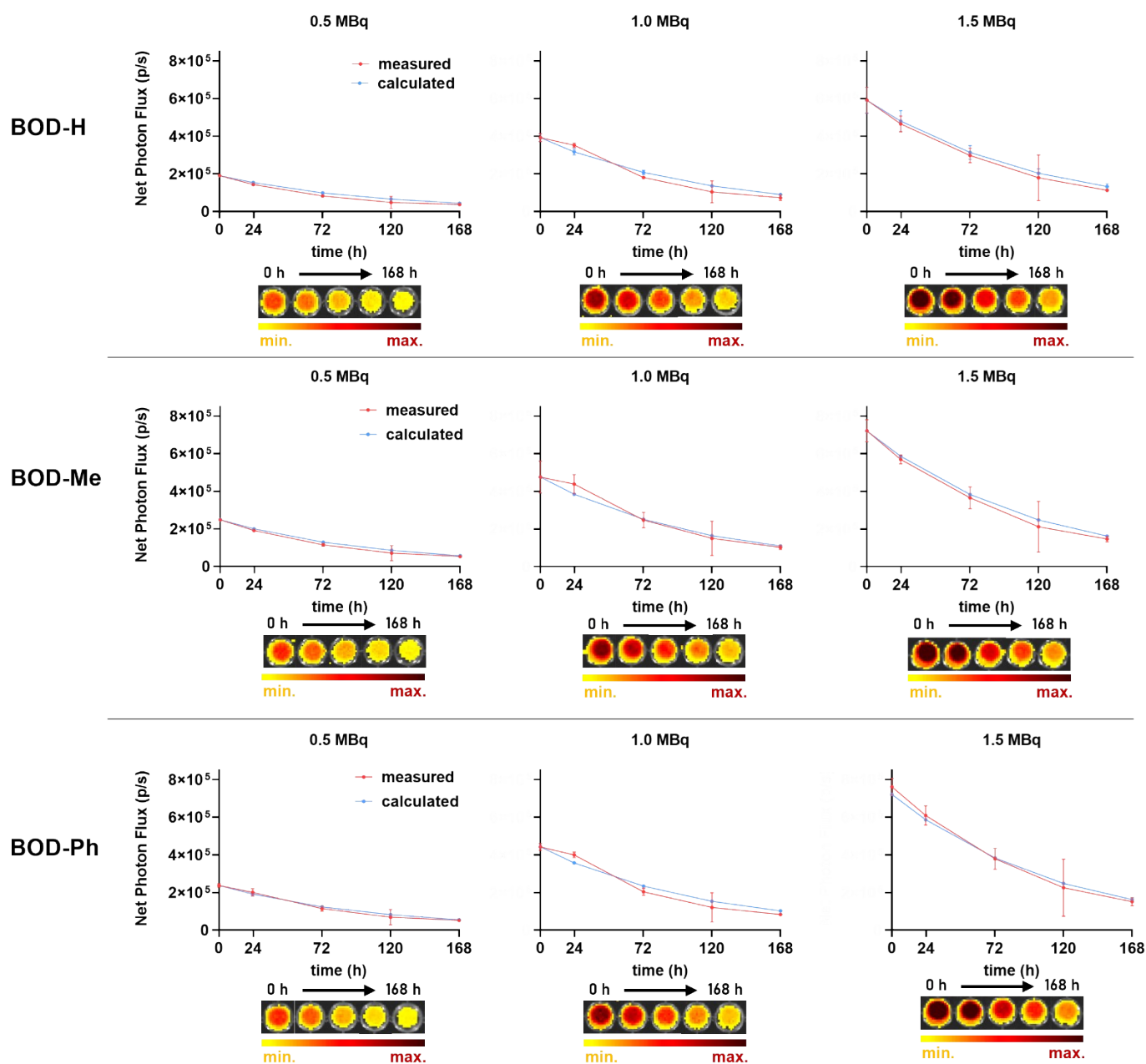


Fig. S4. Monitoring SCIFI intensity over 168 h with varying  $^{89}\text{Zr}$  activities at constant dye concentrations for A BOD-H, B BOD-Me, C BOD-Ph. 0.5, 1.0 or 1.5 MBq of  $^{89}\text{Zr}$  was added to 150  $\mu\text{M}$  of each BODIPY in triplicate, 3.33, 6.67 & 10 kBq/ $\mu\text{M}$ , respectively. Net photon flux (p/s) values (red lines) were measured at 0, 24, 72, 120 and 168 h using open filter. See supplementary table 4 for full tabulated data. Theoretical net photon flux (p/s) values (blue lines) were calculated based on the net photon flux (p/s) values for each BODIPY at 0 h and the decay half-life of  $^{89}\text{Zr}$ . Error bars = SD. Inset images show representative open filter IVIS images of sample wells for each  $^{89}\text{Zr}$  activity at 0, 24, 72, 120 and 168 h.

#### Supplementary Fig. 4 – Decay analysis of BODIPY solutions with increasing $^{89}\text{Zr}$ activity

Supplementary Table 4 – Full range of photon flux decay data for increasing <sup>89</sup>Zr activity

BODIPY	<sup>89</sup> Zr Activity	Time (h)	Average Photon Flux (p/s) ± SD (p/s)
BOD-H	0.5 MBq	0	1.91E+05 ± 3.97E+03
		24	1.44E+05 ± 6.31E+03
		72	8.28E+04 ± 2.18E+03
		120	4.82E+04 ± 3.25E+04
		168	3.70E+04 ± 2.29E+03
	1.0 MBq	0	3.93E+05 ± 2.10E+04
		24	3.52E+05 ± 1.17E+04
		72	1.81E+05 ± 5.60E+03
		120	1.04E+05 ± 5.85E+04
		168	7.34E+04 ± 1.40E+04
	1.5 MBq	0	5.91E+05 ± 6.87E+04
		24	4.66E+05 ± 4.21E+04
		72	2.98E+05 ± 3.87E+04
		120	2.49E+05 ± 3.61E+03
		168	1.13E+05 ± 2.42E+03
BOD-Me	0.5 MBq	0	2.48E+05 ± 6.82E+03
		24	1.92E+05 ± 7.55E+03
		72	1.15E+04 ± 8.67E+03
		120	7.13E+04 ± 4.08E+04
		168	5.37E+04 ± 5.51E+02
	1.0 MBq	0	4.76E+05 ± 8.49E+04
		24	4.39E+05 ± 5.03E+04
		72	2.47E+05 ± 4.11E+04
		120	1.50E+05 ± 9.18E+04
		168	1.02E+05 ± 1.16E+04
	1.5 MBq	0	7.22E+05 ± 5.91E+04
		24	5.70E+05 ± 2.36E+04
		72	3.66E+05 ± 5.80E+04
		120	2.89E+05 ± 2.40E+04
		168	1.47E+05 ± 1.45E+04
BOD-Ph	0.5 MBq	0	2.38E+05 ± 9.28E+03
		24	2.01E+05 ± 1.96E+04
		72	1.15E+05 ± 1.40E+04
		120	6.90E+04 ± 4.04E+04
		168	5.22E+04 ± 1.13E+03
	1.0 MBq	0	4.43E+05 ± 1.78E+04
		24	4.00E+05 ± 1.53E+04
		72	2.04E+05 ± 1.84E+04
		120	1.21E+05 ± 7.82E+04
		168	8.31E+04 ± 2.99E+03
	1.5 MBq	0	7.62E+05 ± 4.45E+04
		24	6.09E+05 ± 5.13E+04
		72	3.79E+05 ± 5.62E+04
		120	3.13E+05 ± 9.97E+03
		168	1.51E+05 ± 2.19E+04

## Supplementary Fig. 5 – Photostability analysis of BODIPY dyes through light-excitation

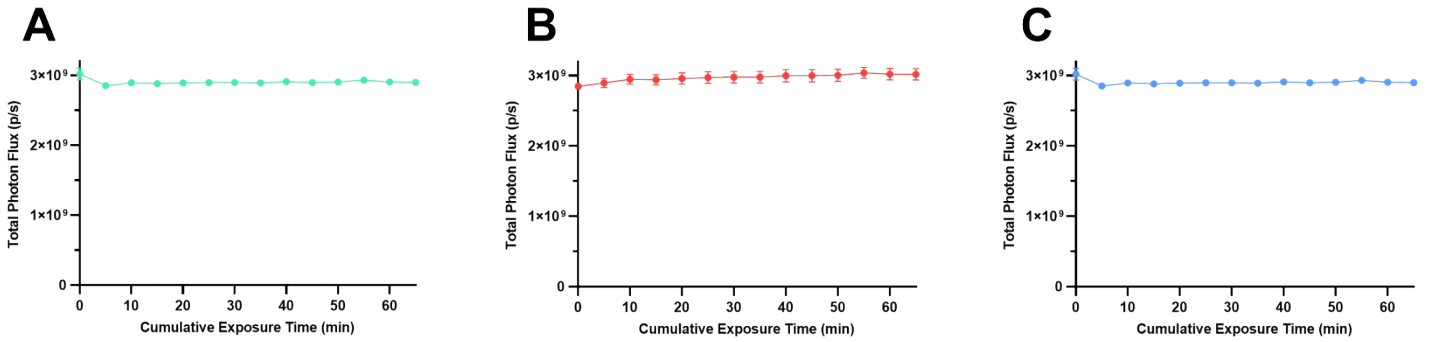


Fig. S5. Photostability of BODIPY dyes following light excitation; A BOD-H, B BOD-Me, C BOD-Ph. Triplicate wells of  $1 \mu\text{M}$  of each BODIPY were repeatedly exposed to 500 nm light for intervals of 5 minutes and total photon flux (p/s) was measured using a 540 nm emission filter on an *in vivo* imaging system. The concentration of  $1 \mu\text{M}$  was chosen to avoid saturation of the light detector in the imaging system, as conventional light excitation yields significantly greater fluorescence intensity compared to Cereknov luminescence, reflected in the y-axis of the figures. Error bars = SD.