Electronic Supplementary Material (ESI) for RSC Advances.

Electronic Supplementary Information

Selective cell elimination *in vitro and in vivo* cell elimination from tissues and tumors using antibodies conjugated with a near infrared phthalocyanine

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Supplemental movies

Supplemental movies S1,S2

Time-lapse Imaging of APC with NIR light *in vitro* 2D cell culture.

Time-lapse sequential images shows ballooning of the cell and rapid membrane damage detected by PI staining after NIR light irradiation in a cell treated with Pan-IR700 (5 sec intervals, total 25 min observation). Flashing light is NIR light irradiation (2 J/cm^2).

Movie S1: DIC time-lapse image of A431-luc-GFP cell treated by APC with NIR light.

Movie S2: Fluorescence of PI time-lapse image of A431-luc-GFP cell treated by APC with NIR light.

Supplemental movies S3,S4

Time-lapse Imaging of APC with NIR light *in vitro* 3D spheroids.

Time-lapse sequential images showed ballooning of the spheroid and rapid membrane damage detected by PI staining after NIR light irradiation in a spheroid treated with Pan-IR700 (5 sec intervals, total 20 min observation). Flashing light is NIR light irradiation (2 J/cm^2).

Movie S3: DIC time-lapse image of A431-luc-GFP spheroid treated by APC with NIR light.

Movie S4: Fluorescence of PI time-lapse image of A431-luc-GFP spheroid treated by APC with NIR light.

Figure S1

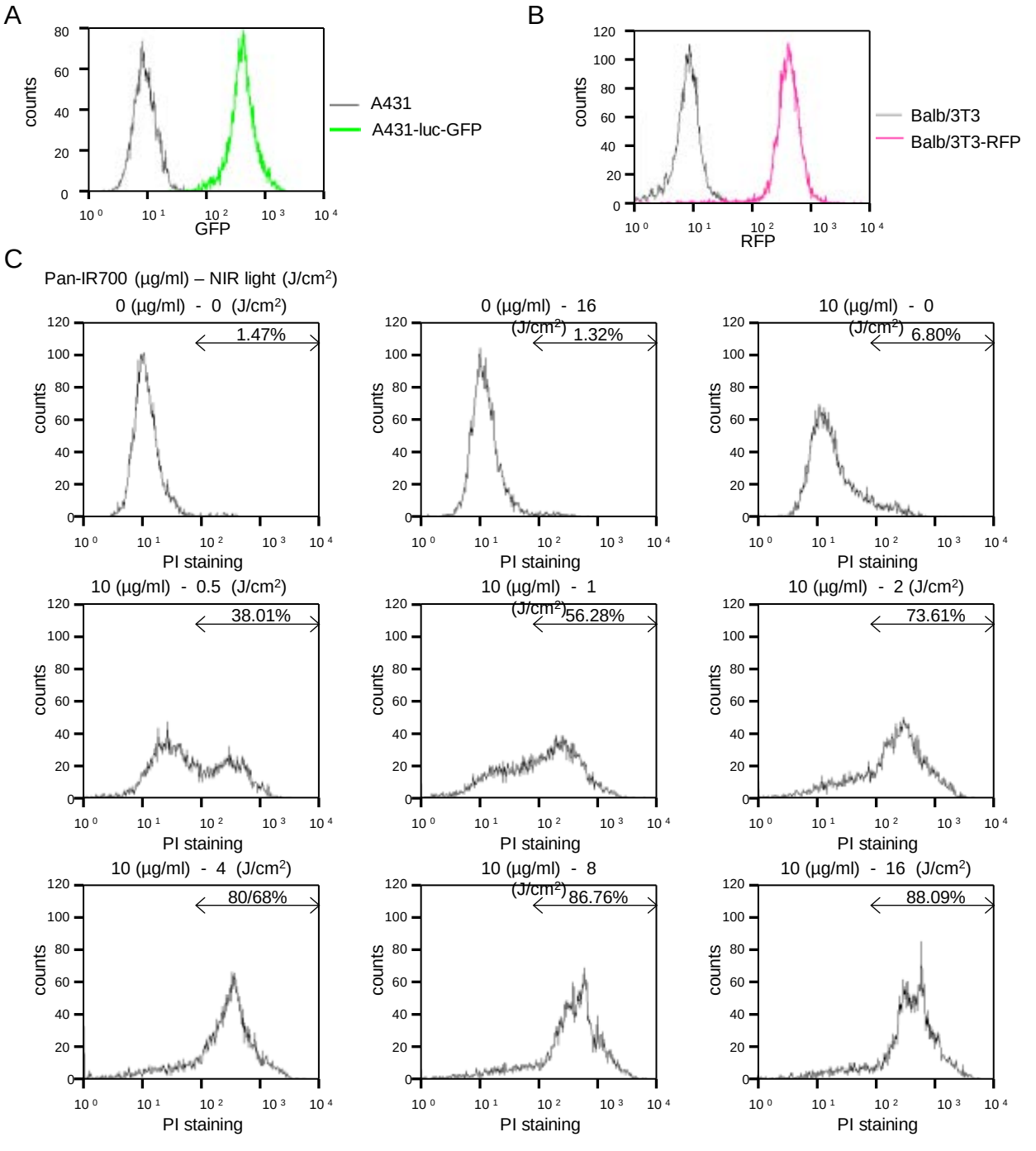

A
counts
GFP
A431
A431-luc-GFP
B
counts
RFP
Balb/3T3
Balb/3T3-RFP
C
Pan-IR700 (μg/ml) – NIR light (J/cm²)
0 (μg/ml) - 0 (J/cm²)
1.47%
0 (μg/ml) - 16 (J/cm²)
1.32%
10 (μg/ml) - 0 (J/cm²)
6.80%
10 (μg/ml) - 0.5 (J/cm²)
38.01%
10 (μg/ml) - 1 (J/cm²)
56.28%
10 (μg/ml) - 2 (J/cm²)
73.61%
10 (μg/ml) - 4 (J/cm²)
80/68%
10 (μg/ml) - 8 (J/cm²)
86.76%
10 (μg/ml) - 16 (J/cm²)
88.09%
PI staining

Figure S1

D

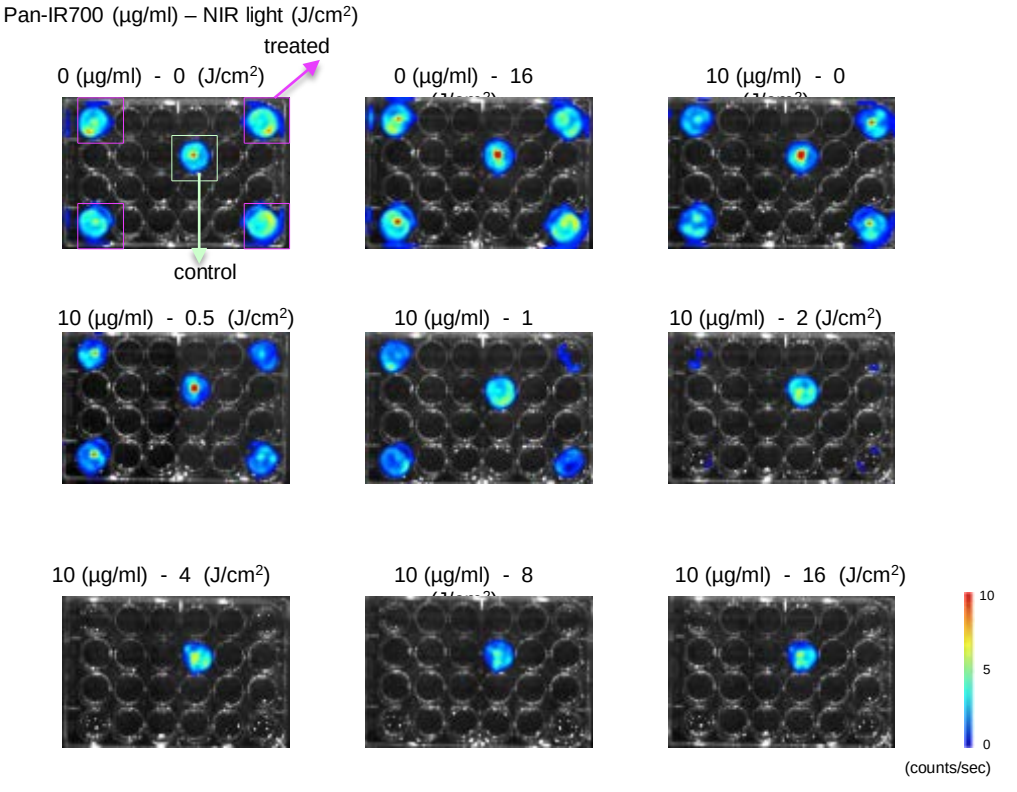

Pan-IR700 (µg/ml) – NIR light (J/cm2)
treated
0 (µg/ml) - 0 (J/cm2)
control
0 (µg/ml) - 16
10 (µg/ml) - 0
10 (µg/ml) - 0.5 (J/cm2)
10 (µg/ml) - 1
10 (µg/ml) - 2 (J/cm2)
10 (µg/ml) - 4 (J/cm2)
10 (µg/ml) - 8
10 (µg/ml) - 16 (J/cm2)
10
5
0
(counts/sec)

Figure S1

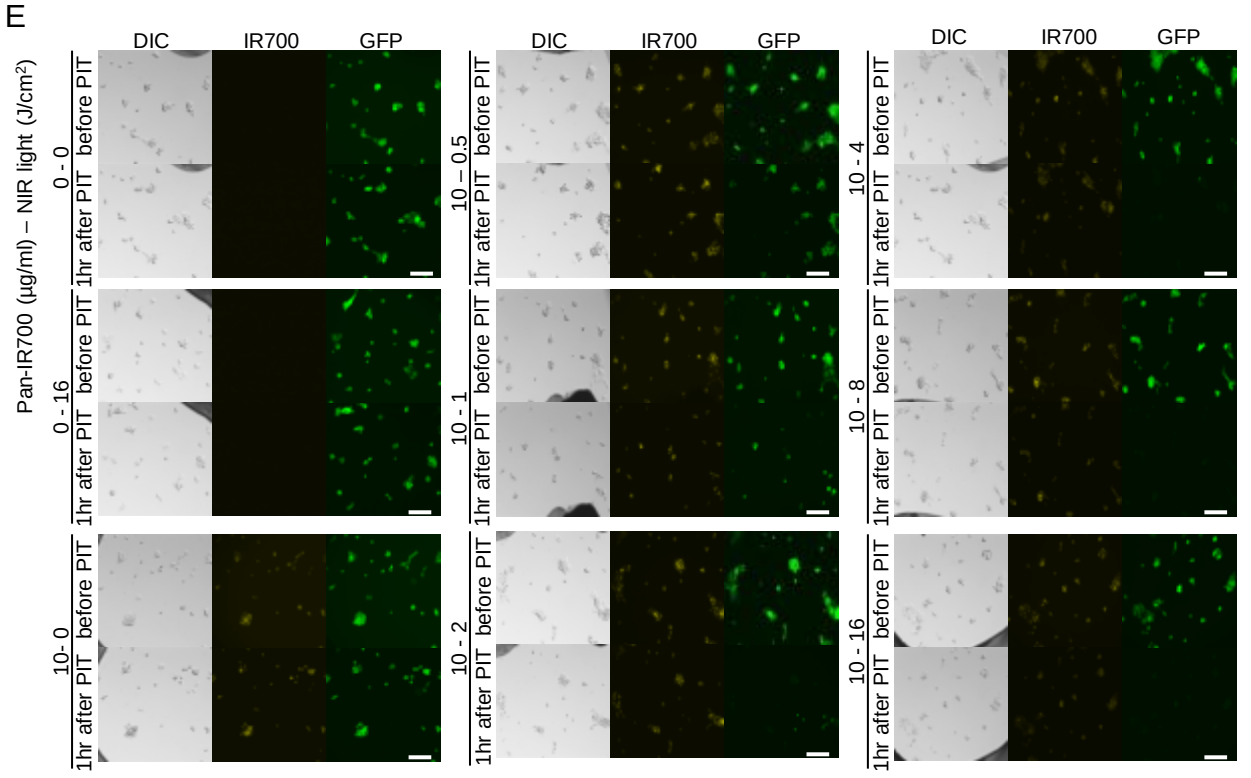

E
Pan-IR700 (μg/ml) – NIR light (J/cm²)
DIC
IR700
GFP
0 - 0
0 - 16
10- 0
10 – 0.5
10 - 1
10 - 2
10 - 4
10 - 8
10 - 16
1hr after PIT
before PIT

Figure S1

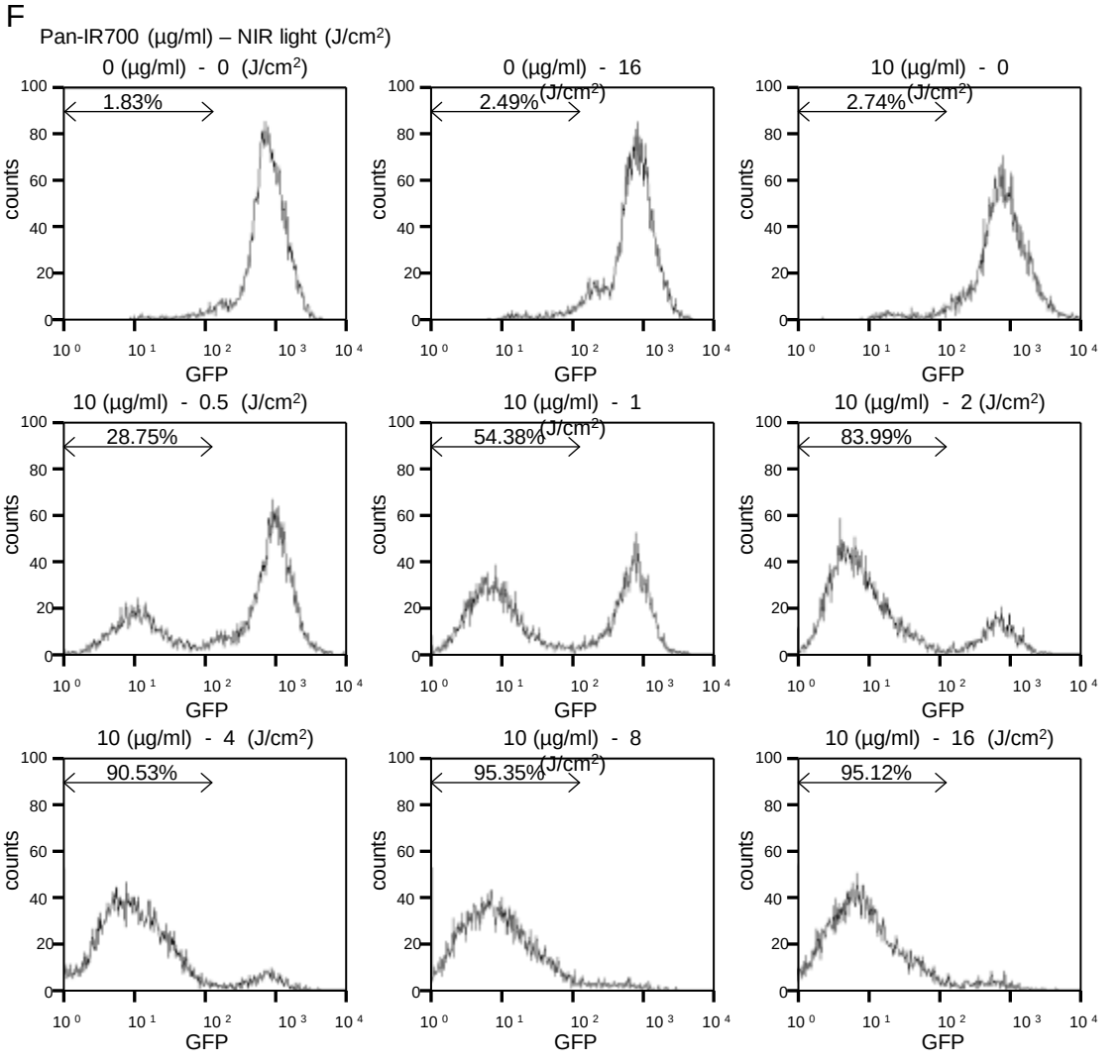

F
Pan-IR700 (μg/ml) – NIR light (J/cm²)
0 (μg/ml) - 0 (J/cm²)
1.83%
0 (μg/ml) - 16 (J/cm²)
2.49%
10 (μg/ml) - 0 (J/cm²)
2.74%
10 (μg/ml) - 0.5 (J/cm²)
28.75%
10 (μg/ml) - 1 (J/cm²)
54.38%
10 (μg/ml) - 2 (J/cm²)
83.99%
10 (μg/ml) - 4 (J/cm²)
90.53%
10 (μg/ml) - 8 (J/cm²)
95.35%
10 (μg/ml) - 16 (J/cm²)
95.12%
counts
GFP

Figure S1

APC with NIR light effect on A431 cell line.

(A) A431 cells were stably transfected with luciferase and GFP (both on the same plasmid) as confirmed by FACS. (B) Balb/3T3 cells were stably transfected with RFP as confirmed by FACS. (C)Quantification of NIR-PIT effect on 2D cultures of A431-luc-GFP cells by FACS analysis. Membrane damage and necrosis induced by NIR-PIT was measured by dead cell count using PI staining on FACS. Cell killing increased in a NIR-light dose-dependent manner.(D)Quantification of APC with NIR light effect on 2D culture of A431-luc-GFP cells by luciferase activity.

Bioluminescence in A431-luc-GFP cells was measured as relative light unit (RLU), and was decreased in a NIR-light dose-dependent manner (1 hr after APC with NIR light). (E) GFP-fluorescence decreased at 1 hr after APC with NIR light in 2D cell culture. Diminishing GFP-fluorescence intensity at 1 hr after APC with NIR light occurred in a NIR-light dose-dependent manner. The black line at the right upper corner was the marker to ensure observation took place consistently. (F) Decrease in GFP-fluorescence at 1 hr after NIR-PIT evaluated with flow cytometry. GFP fluorescence intensity decreased after APC with NIR light in a NIR-light dose-dependent manner as measured by FACS.

Figure S2

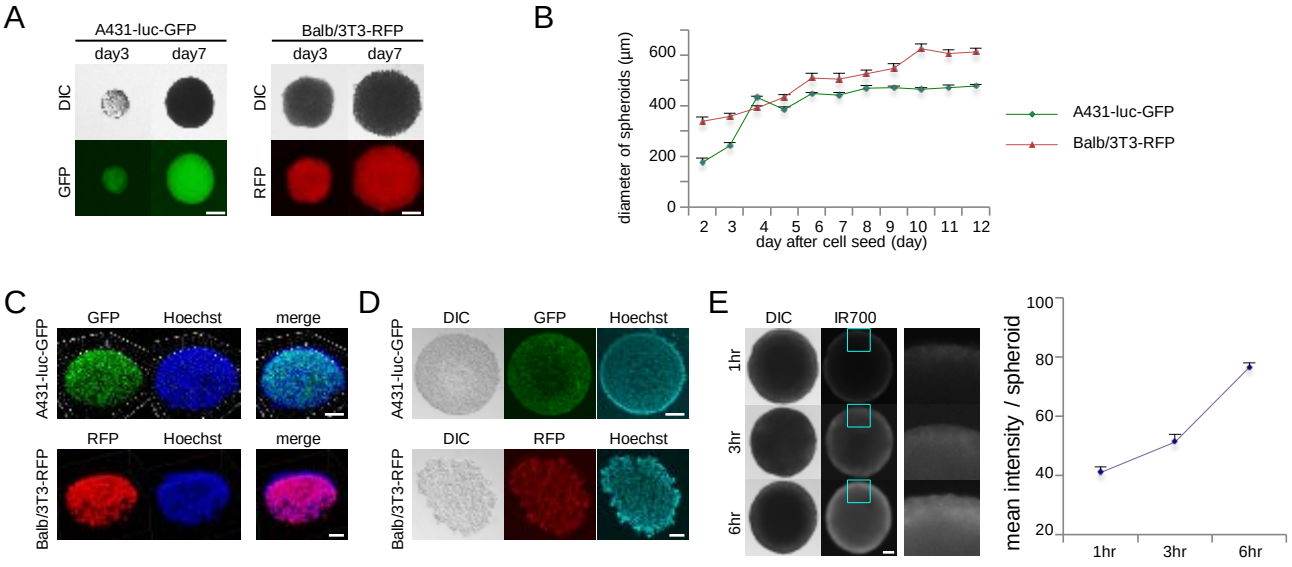
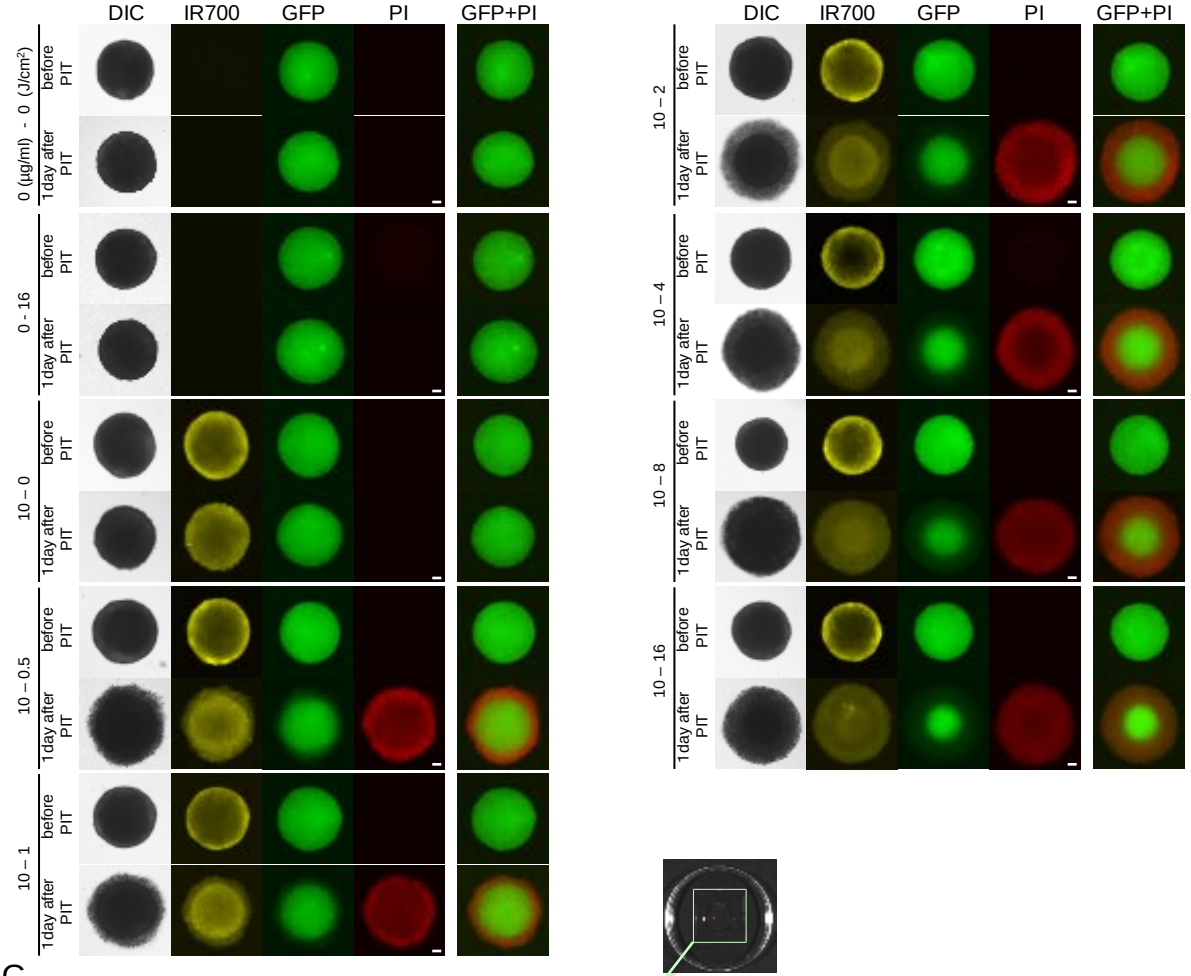

A
A431-luc-GFP
day3
day7
Balb/3T3-RFP
day3
day7
DIC
GFP
DIC
RFP
B
diameter of spheroids (μm)
600
400
200
0
2 3 4 5 6 7 8 9 10 11 12
day after cell seed (day)
A431-luc-GFP
Balb/3T3-RFP
C
GFP
Hoechst
merge
A431-luc-GFP
RFP
Hoechst
merge
Balb/3T3-RFP
D
DIC
GFP
Hoechst
A431-luc-GFP
DIC
RFP
Hoechst
Balb/3T3-RFP
E
DIC
IR700
1hr
3hr
6hr
mean intensity / spheroid
100
80
60
40
20
1hr
3hr
6hr

Figure S2

F


DIC
IR700
GFP
PI
GFP+PI
0 (μg/ml) - 0 (J/cm²)
0 - 16
10 – 0
10 – 0.5
10 – 1
10 – 2
10 – 4
10 – 8
10 – 16
before PIT
1day after PIT

G

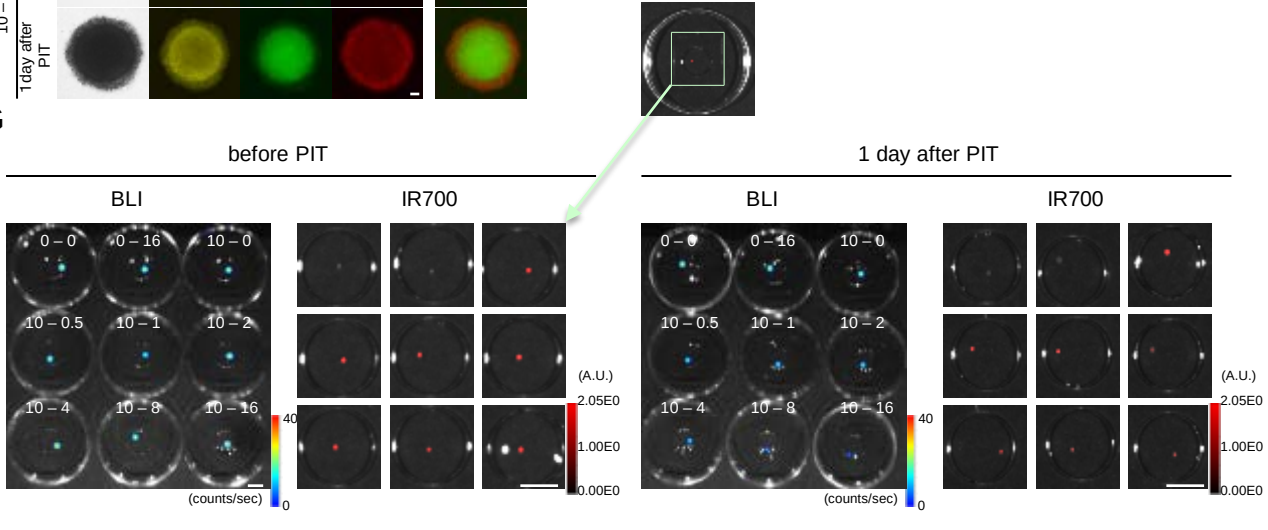
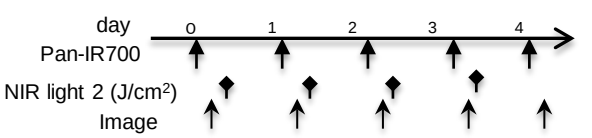
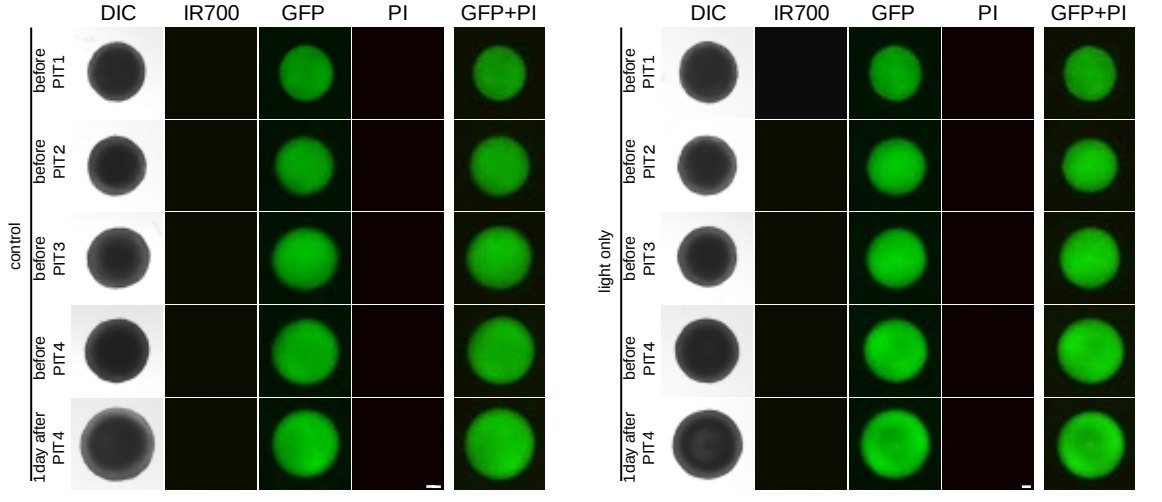

before PIT
1 day after PIT
BLI
IR700
0 – 0
0 – 16
10 – 0
10 – 0.5
10 – 1
10 – 2
10 – 4
10 – 8
10 – 16
(counts/sec)
40
0
(A.U.)
2.05E0
1.00E0
0.00E0

Figure S2

H



I



J

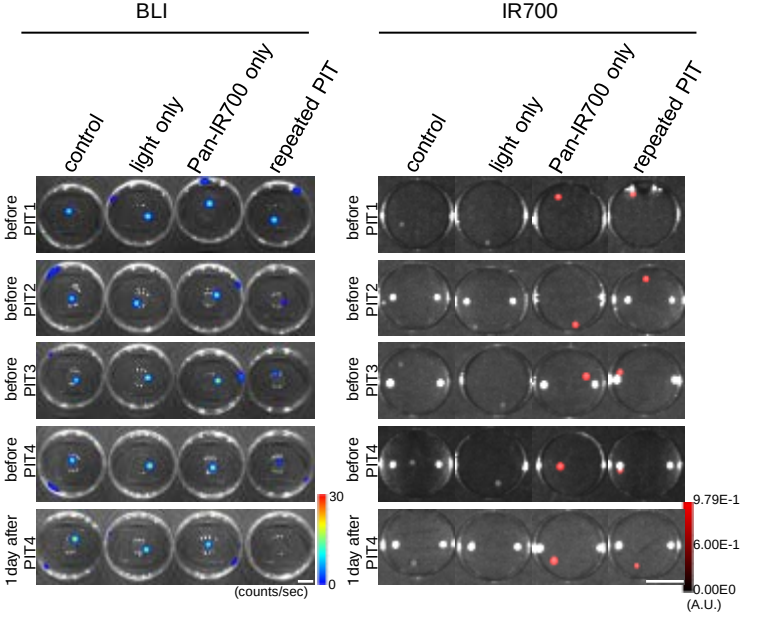


Figure S2

APC with NIR light effect on *in vitro* 3D spheroids.

(A) Characterization of *in vitro* 3D spheroids. Representative image of A431-luc-GFP/ Balb 3T3-RFP 3D spheroids. Bar = 200 μm. (B) 3D spheroids grew to around 500 μm (n = 10). (C) 3D reconstruction image of a 3D spheroid at day 7. Bar = 100 μm. (D) Frozen section of 3D spheroid. Cells accumulate within the core of the spheroid. Bar = 100 μm. (E) Pan-IR700 permeates centrally in a time-dependent manner (mean intensity of IR700 fluorescence in a spheroid)(n = 10). (F) Evaluation of APC with NIR light effect on *in vitro* 3D spheroids. Day 7 3D spheroid at after 6hr incubation with Pan-IR700, before and 1 day after irradiation of NIR light. Necrotic cell death was observed 1 day after NIR light (stained by PI). Bar = 100 μm. GFP-fluorescence intensity decreased and the spheroid decreased in size ("peeling") in a light dose dependent manner. (G) Bioluminescence imaging (BLI) of a spheroid in glass-bottom dish demonstrated that luciferase activity in A431-luc-GFP 3D spheroids decreased in a NIR-light dose-dependent manner at 1 day after APC with NIR light . Bar = 5 mm. Macroscopic view of IR700 fluorescence was also demonstrated (Pearl Imager). (H) The APC with NIR light regimen incorporating repeated NIR light exposures is shown. (I) Effects of repeated NIR-PIT on 3D spheroids. Day 7 A431-luc-GFP 3D spheroids were divided into 4 groups as shown. Bar = 100 μm. (J) Bioluminescence imaging (BLI) of each group demonstrated that luciferase activity decreased after repeated NIR-PIT. Bar = 5 mm. Macroscopic view of IR700 fluorescence was also demonstrated (by Pearl Imager).

Figure S3

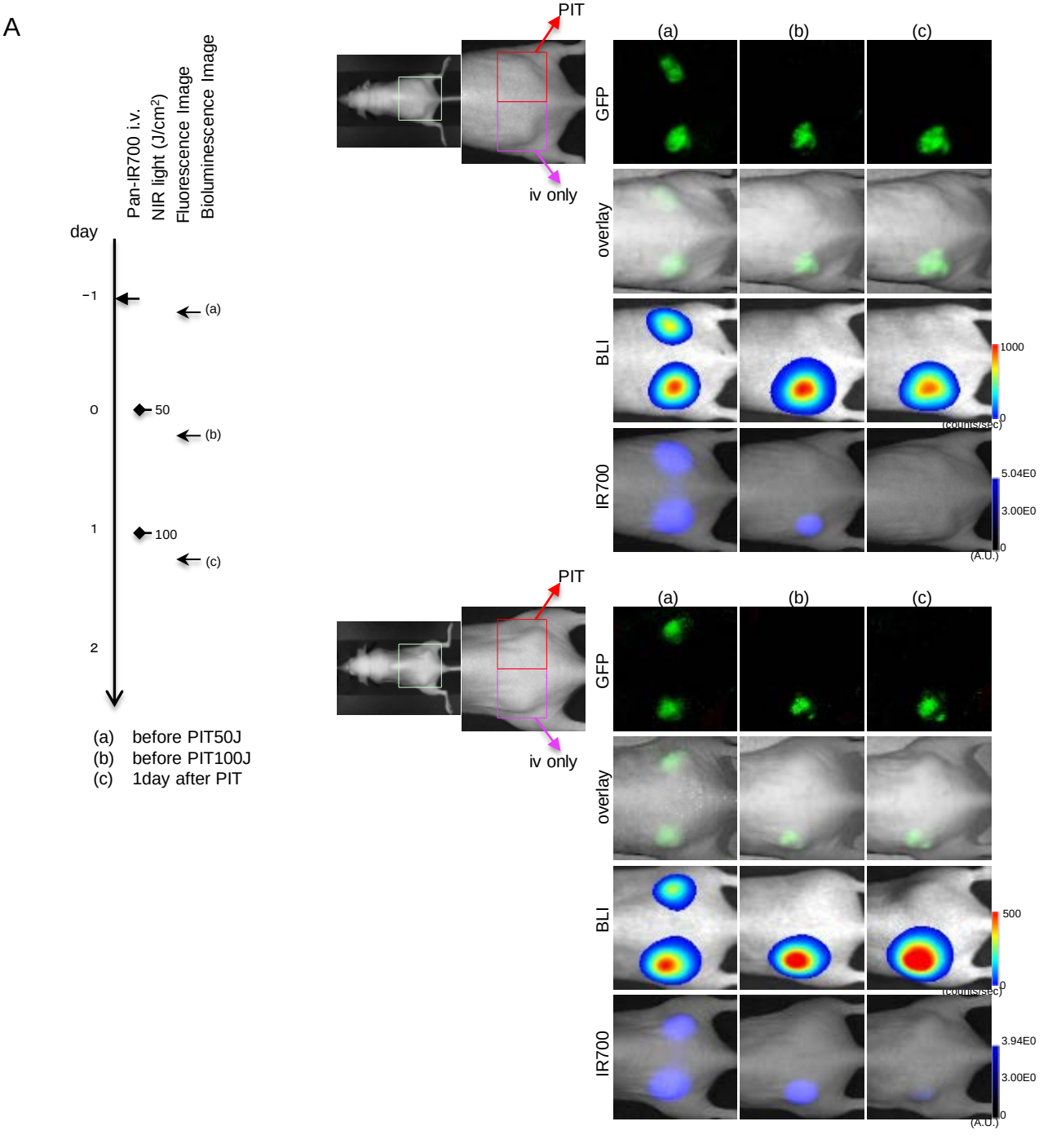
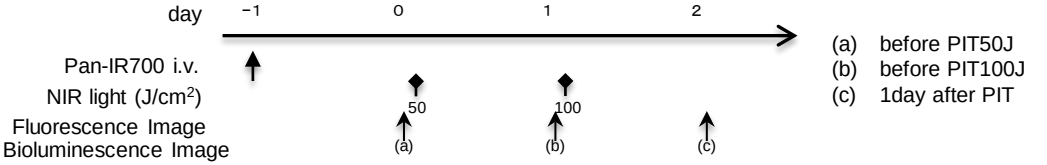

A
Pan-IR700 i.v.
NIR light (J/cm²)
Fluorescence Image
Bioluminescence Image
day
−1
0
1
2
(a)
50
(b)
100
(c)
(a) before PIT50J
(b) before PIT100J
(c) 1day after PIT
PIT
iv only
(a)
(b)
(c)
GFP
overlay
BLI
IR700
1000
0
(counts/sec)
5.04E0
3.00E0
0
(A.U.)
PIT
iv only
(a)
(b)
(c)
GFP
overlay
BLI
IR700
500
0
(counts/sec)
3.94E0
3.00E0
0
(A.U.)

Figure S3

B


day
−1
0
1
2
Pan-IR700 i.v.
NIR light (J/cm2)
50
100
Fluorescence Image
Bioluminescence Image
(a)
(b)
(c)
(a) before PIT50J
(b) before PIT100J
(c) 1day after PIT

C

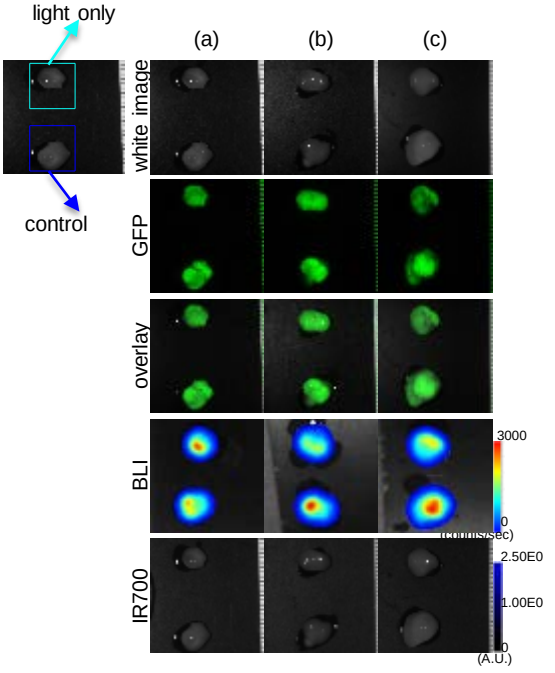

light only
control
(a)
(b)
(c)
white image
GFP
overlay
BLI
IR700
3000
0
(counts/sec)
2.50E0
1.00E0
0
(A.U.)

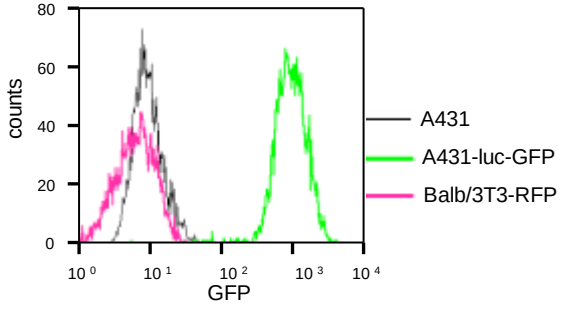
Figure S3

APC with NIR light effect on *in vivo* A431-luc-GFP flank tumor.

(A) *In vivo* GFP/ IR700 fluorescence imaging and BLI of bilateral flank tumors in two additional mice. The tumor treated with NIR-PIT demonstrated loss of both GFP fluorescence and bioluminescence after NIR-PIT. (B) The APC with NIR light regimen incorporating repeated NIR light exposures is shown. (C) Demonstration of APC with NIR light effect on ex *vivo* A431-luc-GFP flank tumor. *Ex vivo* GFP/ IR700 fluorescence imaging and BLI of a flank tumor in response to APC with NIR light .

Figure S4

A


80
60
40
20
0
counts
10 0
10 1
10 2
10 3
10 4
GFP
A431
A431-luc-GFP
Balb/3T3-RFP

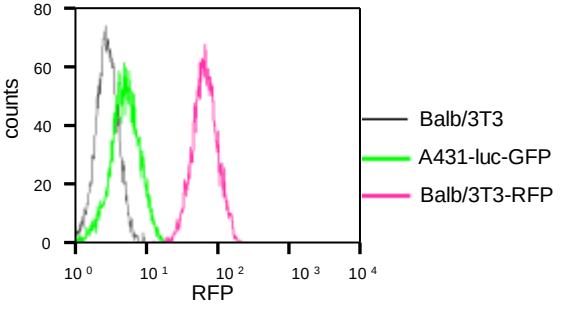

80
60
40
20
0
counts
10 0
10 1
10 2
10 3
10 4
RFP
Balb/3T3
A431-luc-GFP
Balb/3T3-RFP

Figure S4

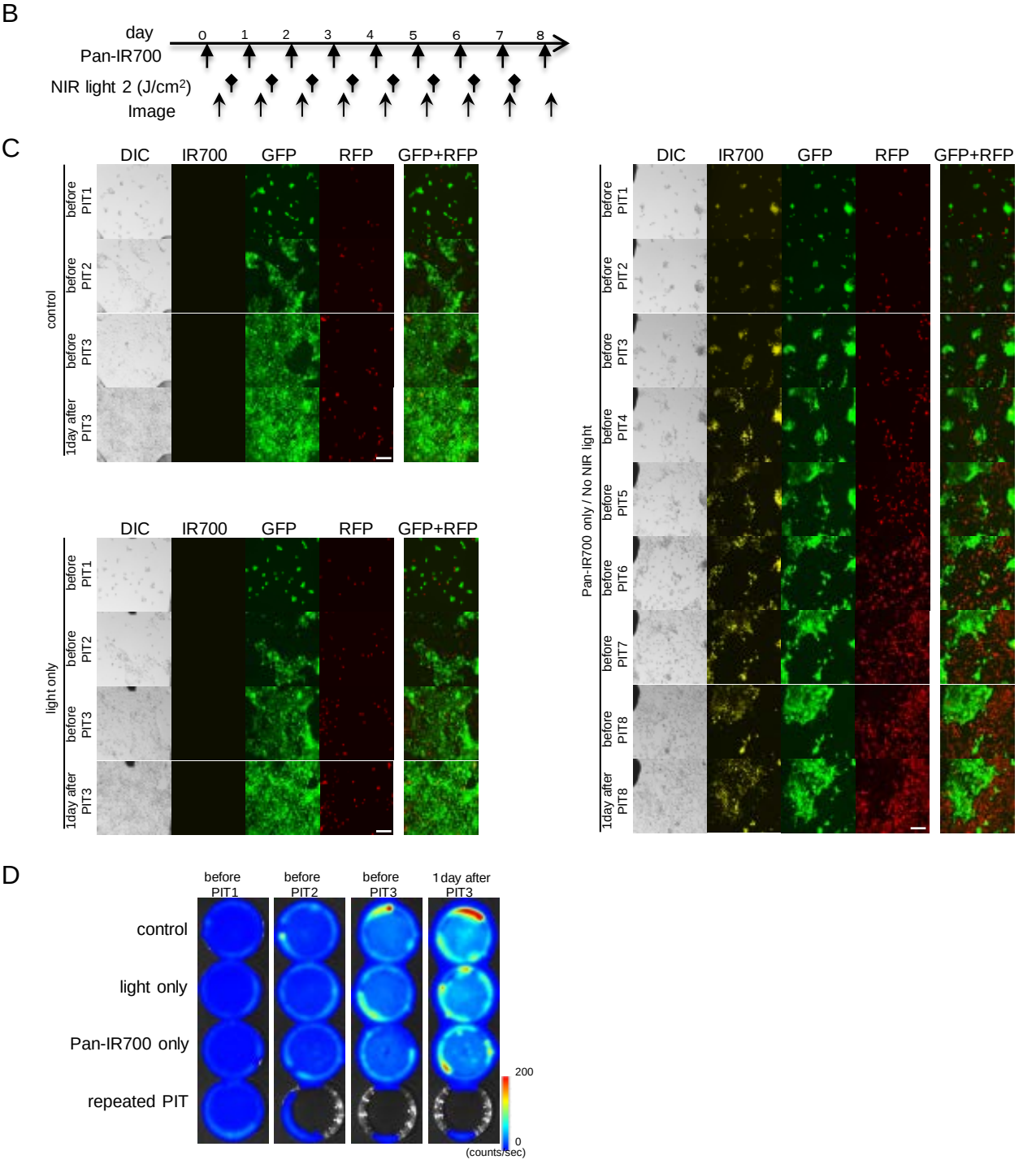

B
day
Pan-IR700
NIR light 2 (J/cm²)
Image
0 1 2 3 4 5 6 7 8
C
DIC IR700 GFP RFP GFP+RFP
control
light only
Pan-IR700 only / No NIR light
before PIT1
before PIT2
before PIT3
1day after PIT3
before PIT4
before PIT5
before PIT6
before PIT7
before PIT8
1day after PIT8
D
before PIT1
before PIT2
before PIT3
1day after PIT3
control
light only
Pan-IR700 only
repeated PIT
200
0
(counts/sec)

Figure S4

Target cell killing in 2D cell culture.

(A) Confirmation of selective/ specific fluorescence in stable cells and specific killing effect of APC with NIR light. FACS demonstrates sorting of the two cell lines (A431 and Balb/3T3) by their GFP and RFP. (B) The APC with NIR light regimen incorporating repeated NIR light exposures is shown. (C) Repeated APC with NIR light completely eliminated target cells with no damage to non-target cells, until non-target cells became confluent. 100:10 ratio of A431-luc-GFP and Balb/3T3-RFP mixed cells were cultured Control group is demonstrated and the black line at edge is a marker to maintain consistent positioning. Bar = 200 μm. (D) BLI of a 35 mm dish demonstrated that luciferase activity in A431-luc-GFP cells progressively decreased after repeated APC with NIR light eventually completely disappearing.

Figure S5

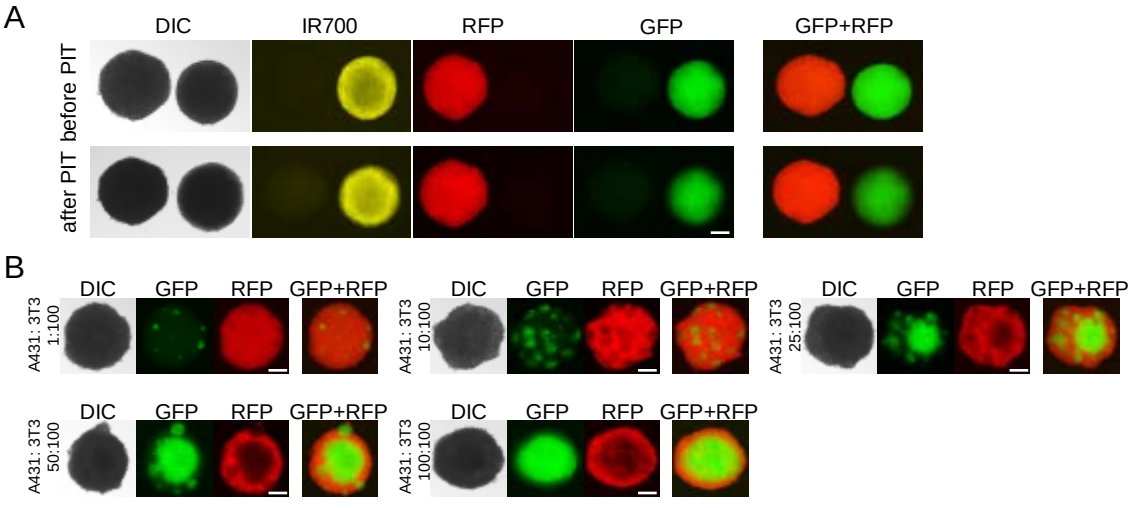

A
DIC
IR700
RFP
GFP
GFP+RFP
before PIT
after PIT
B
DIC
GFP
RFP
GFP+RFP
A431: 3T3
1:100
10:100
25:100
50:100
100:100

Figure S5

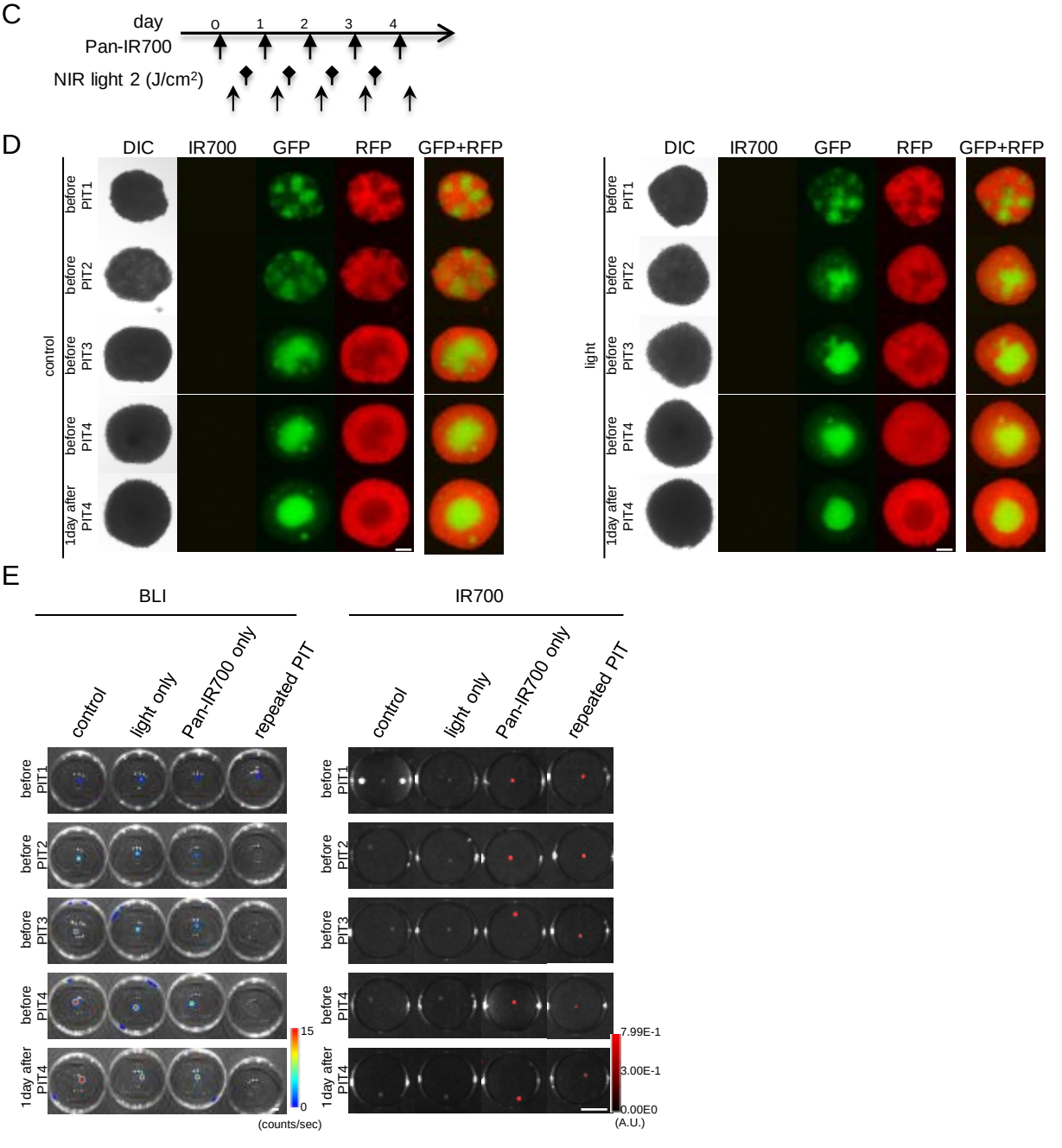
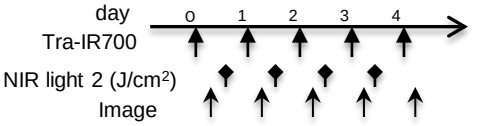

C
day
0
1
2
3
4
Pan-IR700
NIR light 2 (J/cm²)
D
DIC
IR700
GFP
RFP
GFP+RFP
control
light
before PIT1
before PIT2
before PIT3
before PIT4
1day after PIT4
E
BLI
IR700
control
light only
Pan-IR700 only
repeated PIT
15
0
(counts/sec)
7.99E-1
3.00E-1
0.00E0
(A.U.)

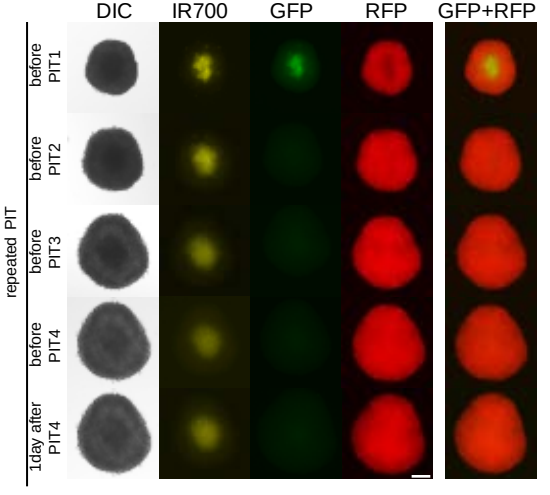
Figure S5

F

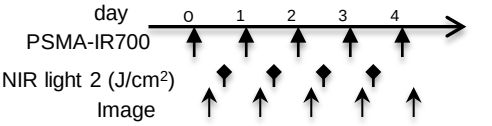


HER2 Target

3T3Her2-luc-GFP
3T3-RFP
mix



G



PSMA Target

PC3-PIP-luc-GFP
3T3-RFP
mix

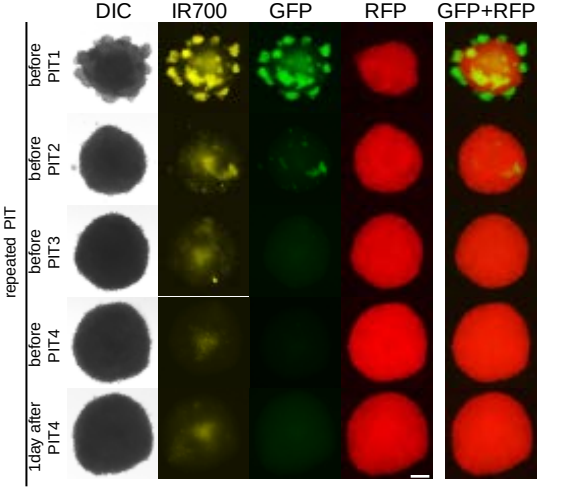


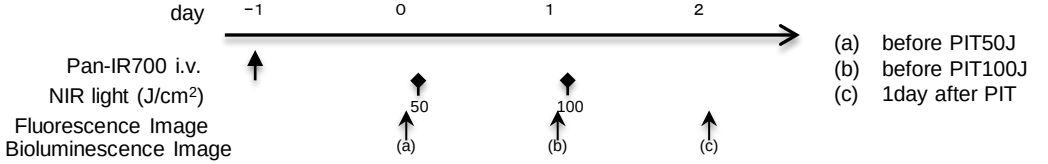
Figure S5

Target cell elimination in 3D mixed cell spheroids.

(A) The effect of APC with NIR light on a spheroid containing A431-luc-GFP cells while no damage is done to the spheroid containing Balb/3T3-RFP cells. Bar = 200 µm. (B) Characterization of various ratios of mixed spheroid at day 7. Bar = 200 µm. (C) Target cell elimination in 3D mixed cell spheroid. Treatment regimen is shown. (D) Repeated NIR-PIT completely eliminated target cells while not damaging non-target cells, in a mixed 3D cell culture. Control group (control and light only) microscopy is shown. Bar = 200 µm. (E) BLI of a spheroid in a glass-bottom dish demonstrated reductions in luciferase activity in mixed 3D spheroids after APC with NIR light eventually leading to complete disappearance. Bar = 5 mm. Macroscopic view of IR700 fluorescence was also demonstrated (Pearl Imager). (F) Target cell (HER2 target and PSMA expressing cells) elimination in 3D spheroids. Regimen of repeat APC with NIR light (2 J/cm^2) is shown above the image. Repeated APC with NIR light exposure completely eliminated HER2 expressing cells while not harming non-target cells. Bar = 200 µm. (G) Regimen of repeat APC with NIR light (2 J/cm^2) shown above the image. Repeat APC with NIR light exposure completely eliminated PSMA targeted cells while not harming non-target cells. Bar = 200 µm.

Figure S6

A


day
−1
0
1
2
Pan-IR700 i.v.
NIR light (J/cm²)
50
100
Fluorescence Image
Bioluminescence Image
(a)
(b)
(c)
(a) before PIT50J
(b) before PIT100J
(c) 1day after PIT

B

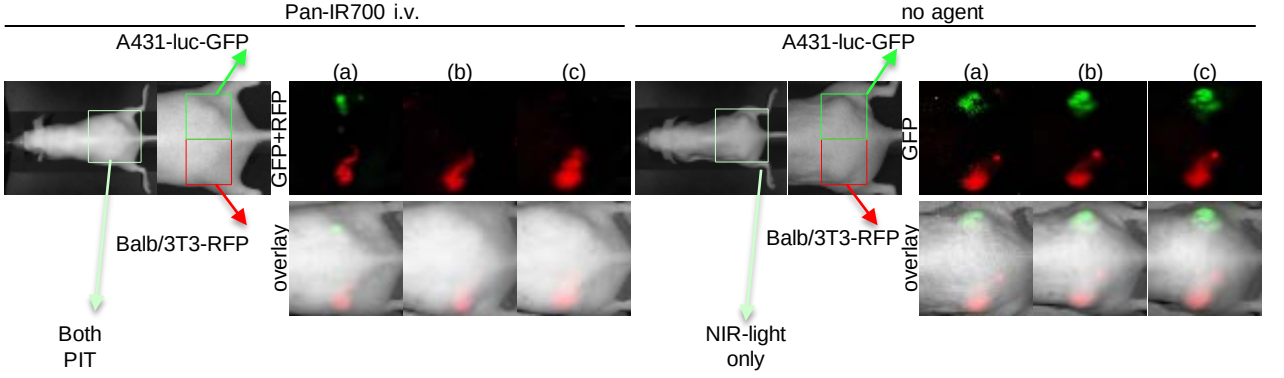

Pan-IR700 i.v.
no agent
A431-luc-GFP
A431-luc-GFP
(a)
(b)
(c)
(a)
(b)
(c)
GFP+RFP
GFP
Balb/3T3-RFP
Balb/3T3-RFP
overlay
overlay
Both
PIT
NIR-light
only

Figure S6

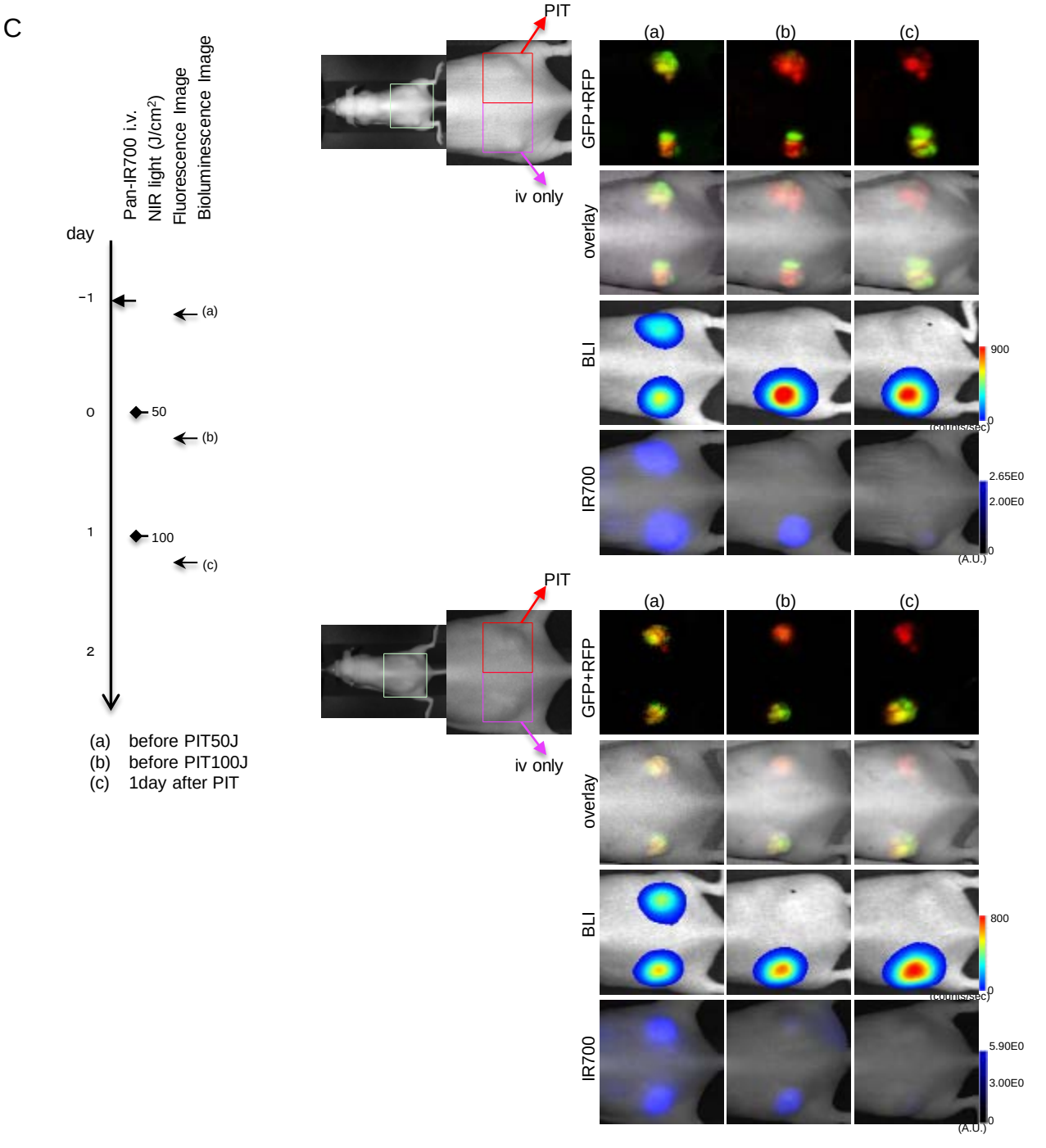
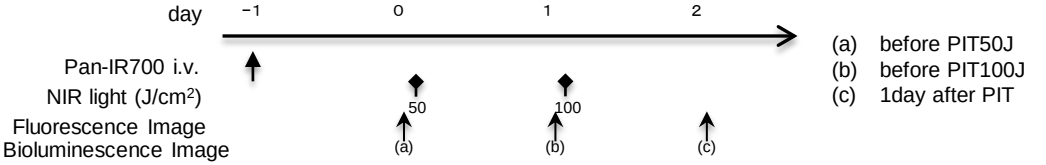

C
Pan-IR700 i.v.
NIR light (J/cm²)
Fluorescence Image
Bioluminescence Image
day
−1
0
1
2
(a)
50
(b)
100
(c)
(a) before PIT50J
(b) before PIT100J
(c) 1day after PIT
PIT
iv only
(a)
(b)
(c)
GFP+RFP
overlay
BLI
IR700
900
0
(counts/sec)
2.65E0
2.00E0
0
(A.U.)
PIT
iv only
(a)
(b)
(c)
GFP+RFP
overlay
BLI
IR700
800
0
(counts/sec)
5.90E0
3.00E0
0
(A.U.)

Figure S6

D


day
−1
0
1
2
Pan-IR700 i.v.
NIR light (J/cm²)
50
100
Fluorescence Image
Bioluminescence Image
(a)
(b)
(c)
(a) before PIT50J
(b) before PIT100J
(c) 1day after PIT

E

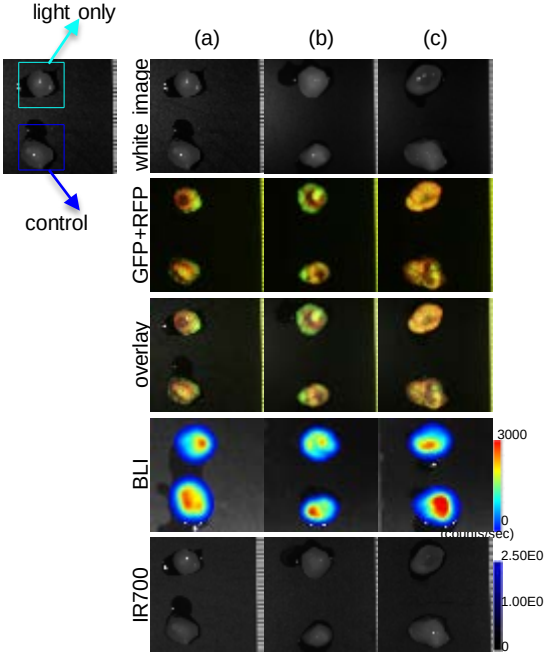

light only
control
(a)
(b)
(c)
white image
GFP+RFP
overlay
BLI
IR700
3000
0
(counts/sec)
2.50E0
1.00E0
0

Figure S6

Target cell elimination from mixed cell tumors *in vivo*.

(A) Regimen of repeat APC with NIR light is shown. (B) APC with NIR light demonstrate response in target tumor but no effect on the non-target tumor. (C) Demonstration of target cell elimination *in vivo*. Repeated APC with NIR light completely eliminated target cells in mixed tumors. *In vivo* GFP/ IR700 fluorescence imaging and BLI of bilateral flank tumor (2 additional mice). The tumor treated by APC with NIR light demonstrated disappearance of both GFP fluorescence and bioluminescence after APC with NIR light . (D) Demonstration of cell elimination on ex *vivo* mixed tumor (control tumor). The APC with NIR light regimen incorporating repeated NIR light exposures is shown. (E) *Ex vivo* GFP/ IR700 fluorescence imaging and BLI of a mixed tumor in response to APC with NIR light . *Ex vivo* images of control tumors are shown.