Radiobiological experiments for tumor volume response correlation

BALB-c/nude mice were engrafted subcutaneously with 5 x 10^6 NCI-H69 cells and at a tumor size of 369 ± 203 mm^3 mice were injected intravenously with 30 MBq [^{177}Lu]Lu-DOTATATE (n = 4 per group). Mice were sacrificed and analyzed at 1 h, 1, 2, 3, 4, 5, 7, 9, 11 and 14 days post injection (p.i.). Organs were put in a gamma-counter for measurement of radioactive uptake and then fixed and embedded in paraffin for later analysis. Uninjected animals were used as control (n = 4).

The biodistribution data from the gamma-counter were analyzed to determine the kinetics of the tumors. The measured activity data as a function of time were fitted with single exponential curves, as indicated by (corrected) Akaike's information criterion, using the least-square regression method.

The cells were immunofluorescent (IF) stained as previously described (6). For the stainings p53 binding protein 1 (53BP1) (Novus Biologicals, NB100-904; 1:500), phosphorylated histone 2AX (γH2AX) (Millipore, JBW301; 1:250), $SSTR_2$ (Abcam, 134152; 1:100) primary antibodies were used. Secondary antibodies used are donkey-anti-rabbit IgG Alexa Fluor 594 (Thermo Fisher, A-11078; 1:500) and donkey-anti-mouse IgG Alexa Fluor 488 (Thermo Fisher; A-11005; 1:500).

Tissue sections of 4 µm were deparaffinized and rehydrated. TUNEL assay was performed using the In Situ Cell Death Detection Kit, Fluorescein (Roche, 11684795910) according to manufacturer's instructions.

In order to analyze tumor growth, another set of BALB-c/nude mice were engrafted subcutaneously with 5 x 10^6 NCI-H69 (n = 8) and injected when tumor volumes reached 697 ± 256 mm^3. The treated group was compared to vehicle injected counterparts (n = 8). Tumor volumes were measured three times per week p.i. Mice were sacrificed when tumor volumes reached the humane endpoint of 2000 mm^3.

Radiobiological experiments for double strand breaks (DSBs) correlation

53BP1 and γH2AX focus formation was imaged with a confocal microscope using Z-stack acquisition. ImageJ was utilized to apply the same local threshold (default for DAPI, MaxEntropy for $SSTR_2$) to all images in order to segment nuclei or quantify DAPI signal and quantify IF signal. Foci were quantified using the Find Maxima function.

G factor derivation

$$G(t) = \frac{2}{D^2}\int_{-\infty}^{\infty} dt\ \frac{dD(t)}{dt}\int_{-\infty}^{t} dt'\ \frac{dD(t')}{dt'}\, e^{-\mu(t-t')}$$

Substituting the dose-rate definition and re-ordering:

$$G(t) = \frac{2}{D(t)^2}\int_0^T dt\ \Big((R_0 - P)e^{(-\lambda_b t)} + P\Big)e^{(-\lambda_p t)}\int_{-\infty}^{t} dt'\ \Big((R_0 - P)e^{(-\lambda_b t')} + P\Big)e^{(-\lambda_p t')}\ e^{-\mu(t-t')}$$
$$= \frac{2}{D^2}\int_0^T dt\ \Big((R_0 - P)e^{-(\lambda_e+\mu)t} + Pe^{-(\lambda_p+\mu)t}\Big)\int_{-\infty}^{t} dt'\ \Big((R_0 - P)e^{(-\lambda_e+\mu)t'} + Pe^{(-\lambda_p+\mu)t'}\Big) =$$

Solving the integral in t':

$$G(t) = \frac{2}{D(t)^2}\int_0^T dt\ \Big((R_0 - P) * e^{-(\lambda_e+\mu)t} + Pe^{-(\lambda_p+\mu)t}\Big)\left(\frac{R_0 - P}{\lambda_e - \mu} - \frac{R_0 - P}{\lambda_e - \mu}\ e^{-(\lambda_e-\mu)t} + \frac{P}{\lambda_p - \mu} - \frac{P}{\lambda_p - \mu}e^{-(\lambda_p-\mu)t}\right) =$$

Using linearity and multiplying:

$$G(t) = \frac{2}{D(t)^2}\int_0^T dt\ \left(\frac{(R_0 - P)^2}{\lambda_e - \mu}\ e^{-(\lambda_e+\mu)t} + \frac{(R_0 - P)P}{\lambda_e - \mu}\ e^{-(\lambda_p+\mu)t} - \frac{(R_0 - P)^2}{\lambda_e - \mu}\ e^{-2\lambda_e t} - \frac{(R_0 - P)P}{\lambda_e - \mu}\ e^{-(\lambda_p+\lambda_e)t} + \frac{(R_0 - P)P}{\lambda_p - \mu}\ e^{-(\lambda_e+\mu)t} + \frac{P^2}{\lambda_p - \mu}\ e^{-(\lambda_p+\mu)t} - \frac{(R_0 - P)P}{\lambda_p - \mu}\ e^{-(\lambda_e+\lambda_p)t} - \frac{P^2}{\lambda_p - \mu}\ e^{-2\lambda_p t}\right) =$$

Solving in t:

$$G(t) = \frac{2}{D(t)^2}\left[\frac{(R_0-P)^2}{(\lambda_e{}^2-\mu^2)}\left(1 - e^{-(\lambda_e+\mu)t}\right) + \frac{(R_0-P)P}{(\lambda_e-\mu)(\lambda_p+\mu)}\left(1 - e^{-(\lambda_p+\mu)t}\right) + \frac{(R_0-P)^2}{2\lambda_e(\mu-\lambda_e)}\left(1 - e^{-2\lambda_e t}\right) - \frac{(R_0-P)P}{(\lambda_e-\mu)(\lambda_p+\lambda_e)}\left(1 - e^{-(\lambda_p+\lambda_e)t}\right) + \frac{(R_0-P)P}{(\lambda_p-\mu)(\lambda_e+\mu)}\left(1 - e^{-(\lambda_e+\mu)t}\right) + \frac{P^2}{(\lambda_p{}^2-\mu^2)}\left(1 - e^{-(\lambda_p+\mu)t}\right) - \frac{(R_0-P)P}{(\lambda_p-\mu)(\lambda_e+\lambda_p)}\left(1 - e^{-(\lambda_e+\lambda_p)t}\right) + \frac{P^2}{2\lambda_p(\mu-\lambda_p)}\left(1 - e^{-2\lambda_p t}\right)\right]$$ and

$$D(t) = \frac{R_0 - P}{\lambda_e}\left(1 - e^{-\lambda_e t}\right) + \frac{P}{\lambda_p}\left(1 - e^{-\lambda_p t}\right)$$

MC input data

Each of the excised tissue section is made of 25 tiles of 640.17x 640.17 µm side. The following steps were implemented in a Python (*1*) program to create, using the ITK (www.itk.org) module, the mhd image file format for the MC input data, i.e. voxelized sources and computational models:

- Crop a 50 pixel frame in each tile to avoid corrections compensating for the vignetting effectjnm (*2*)
- Stitch the 25 tiles together to create a larger tissue section of 3.2 x 3.2 mm side

- Decrease the resolution by means of Lancosz filter
- Create a montage replicating the tissue sections from the previous step
- Apply a low threshold only to the voxelized computational models to discern between tumor cells region (referred to as tumor cells) and remaining healthy tissue (referred to as healthy cells)
- Convert image files into the aforementioned mhd format

The voxelized source is interpreted as a map of emission probabilities for the chosen radionuclide, via a linear translation of each voxel value. The composition of the tumor cell region of the voxelized phantom was defined as in the ICRU Report 46 (*3*), whilst the healthy tissue composition was that of water.

Simulations were carried out on the Dutch national e-infrastructure with the support of SURF Cooperative (4 × 8-core 2.7 GHz Intel Xeon E5-4650 CPUs/node and 256 GB/node), due to the memory requirement of these calculations.

Template matching

First, the templates and large images were modified to have the same resolution of 0.625 μm/pixel. Then, we adopted a sliding window algorithm computing normalized pixel value histograms in regions sized as the template for each pixel belonging to the test image (*4*,*5*). The similarity between the local histograms within the large image (test image) and the template image was computed through a chi-squared based distance metric (Supplemental Figure 2) and displayed with a color map.

The marked matching areas were used to identify the areas most likely to present high level of DSB damage for comparison with absorbed dose (0-2days) and dose rate maps at day 2.

REFERENCES

1. Python Software Foundation. Python Language Reference, version 3.6.3.

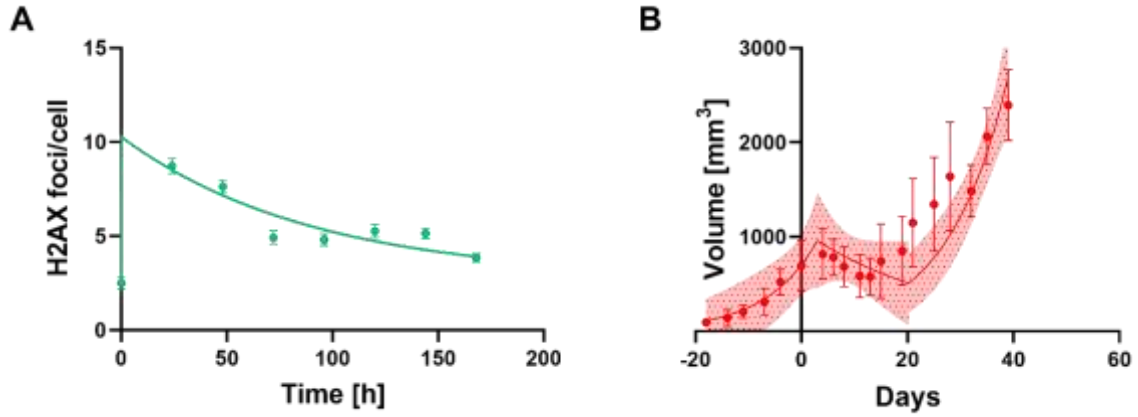
2. Peng T, Thorn K, Schroeder T, et al. A BaSiC tool for background and shading correction of optical microscopy images. *Nat Commun*. 2017;8:1-7.

3. White DR, Griffith R V., Wilson IJ. ICRU Report 46: Photon, Electron, Proton and Neutron Interaction Data for Body Tissues.; 1992.

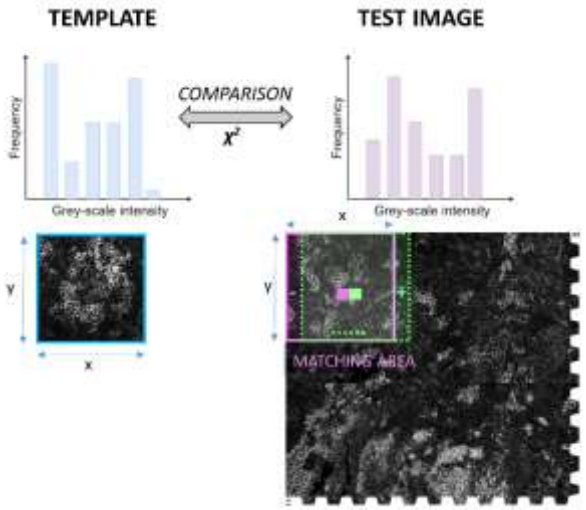
4. Perreault S, Hébert P. Median filtering in constant time. *IEEE Trans Image Process*. 2007;16:2389-2394.

5. Porikli F. Integral histogram: a fast way to extract histograms in Cartesian spaces. *Proc - 2005 IEEE Comput Soc Conf Comput Vis Pattern Recognition*, CVPR 2005. 2005;I:829-837.

6. Nonnekens J, van Kranenburg M, Beerens CE, et al. Potentiation of peptide receptor radionuclide therapy by the PARP Inhibitor Olaparib. *Theranostics*. 2016; 6: 1821-32

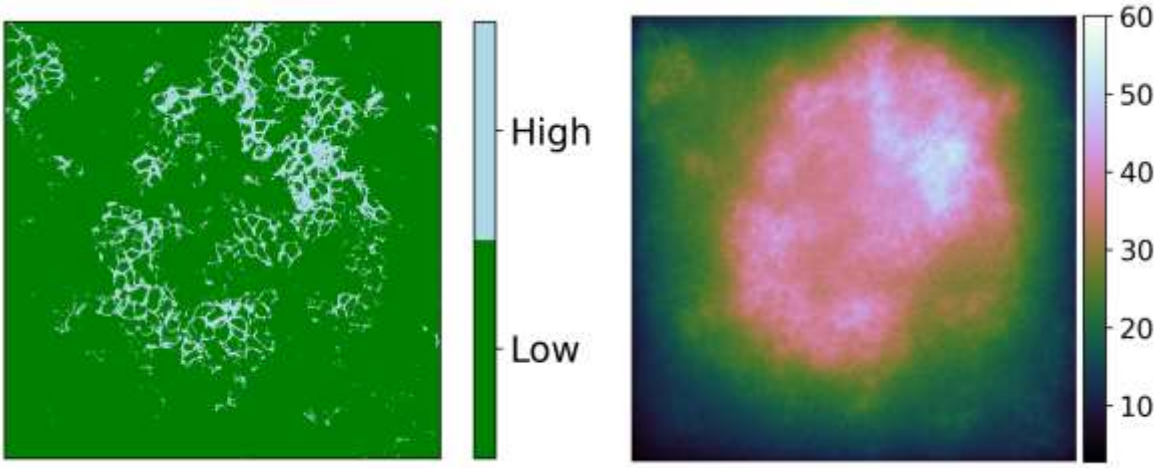


SUPPLEMENTAL FIGURE 1. Experimental radiobiological parameters (11). (2A) *In vitro* quantification of γH2AX foci/cell over time to determine repair rate. The error bars indicate standard error of the mean (SEM). (2B) Tumor growth curve to determine repopulation rate. The error bars indicate 1 standard deviation (SD).

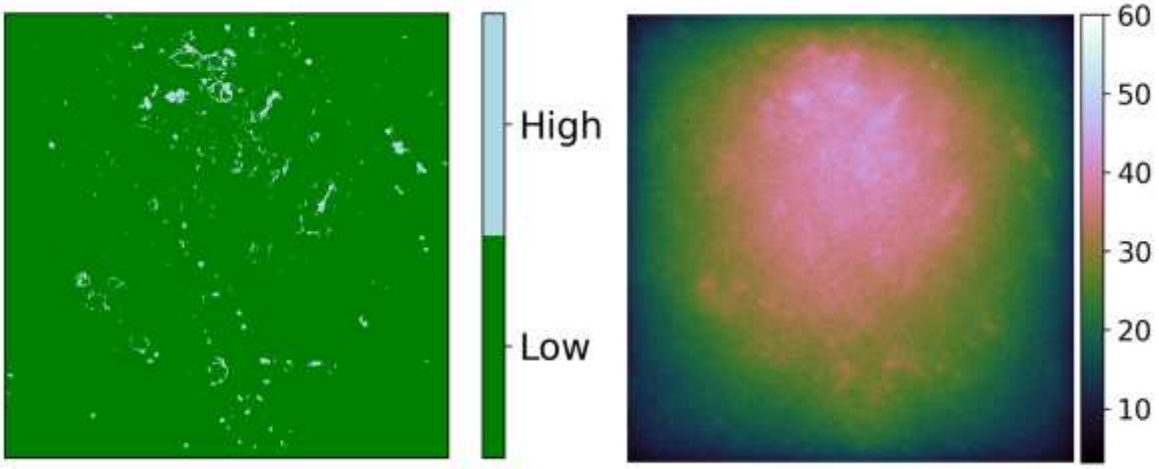


SUPPLEMENTAL FIGURE 2. Schematic representation of template matching algorithm. The template histogram is indicated in light blue, whilst the histogram of the current window centered on the analyzed pixel is indicated in purple. An example of the window movement (towards the right) is shown in green. The window will slide over all the pixels composing the test image and register their similarity score (χ2).

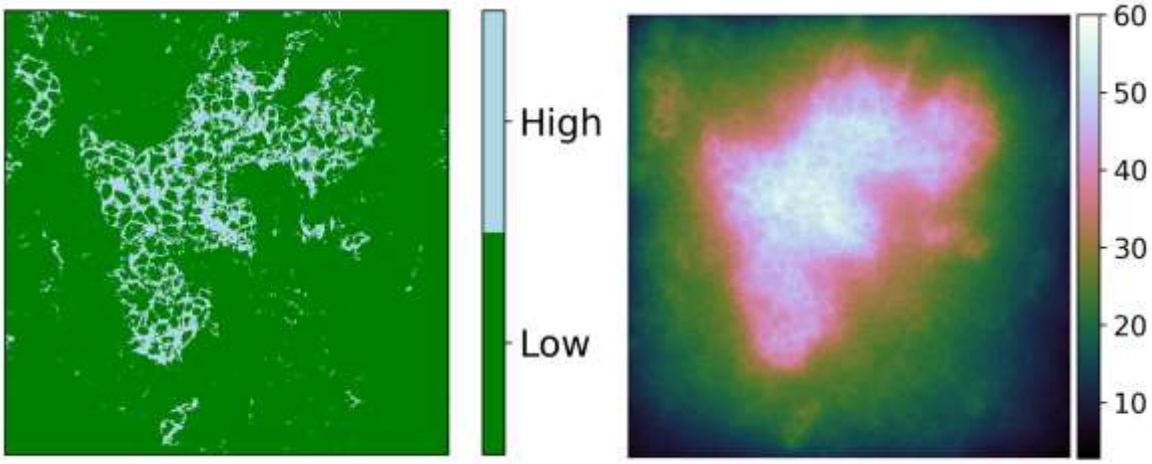
Tissue section B1 "high" $SSTR_2$ expression



Tissue section B1 "low" $SSTR_2$ expression



Tissue section B2 "high" $SSTR_2$ expression



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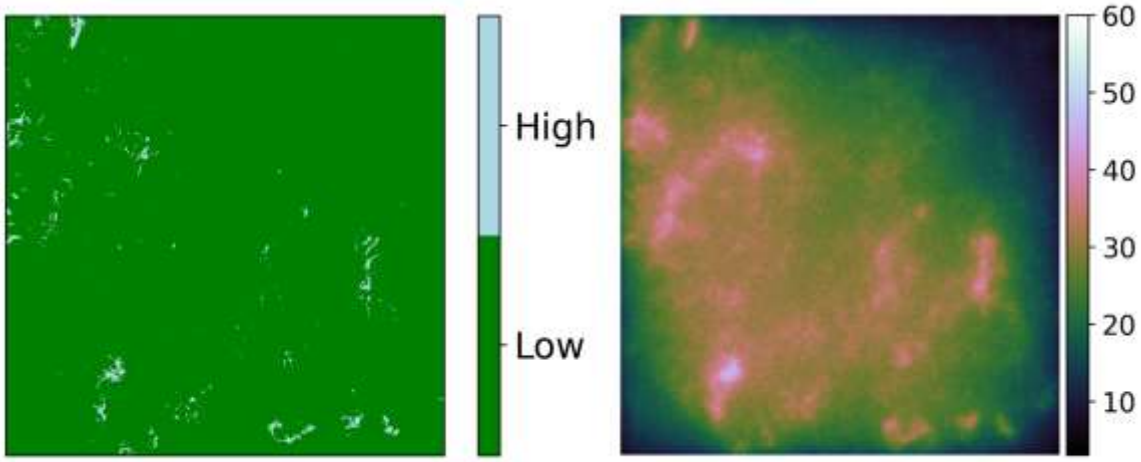
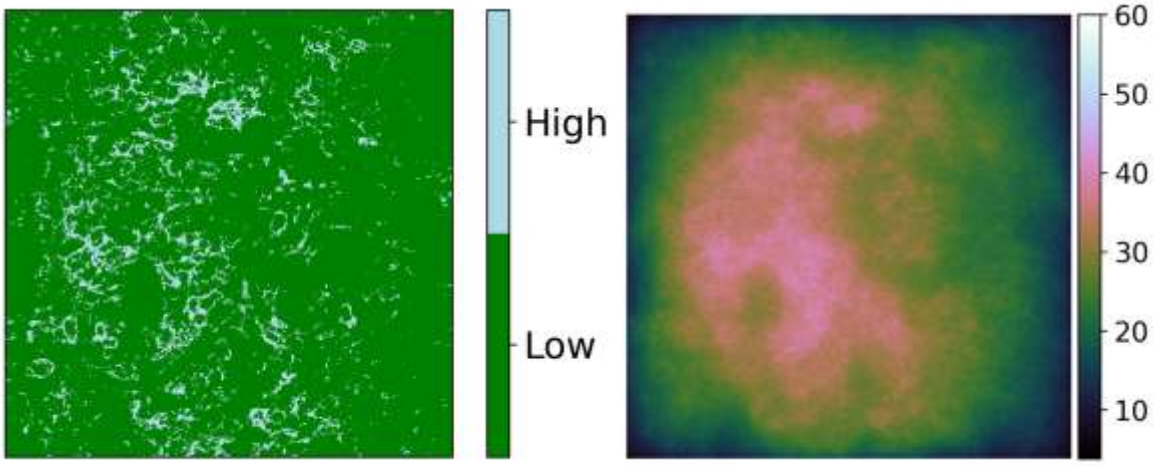


Tissue section B3 "high" $SSTR_2$ expression



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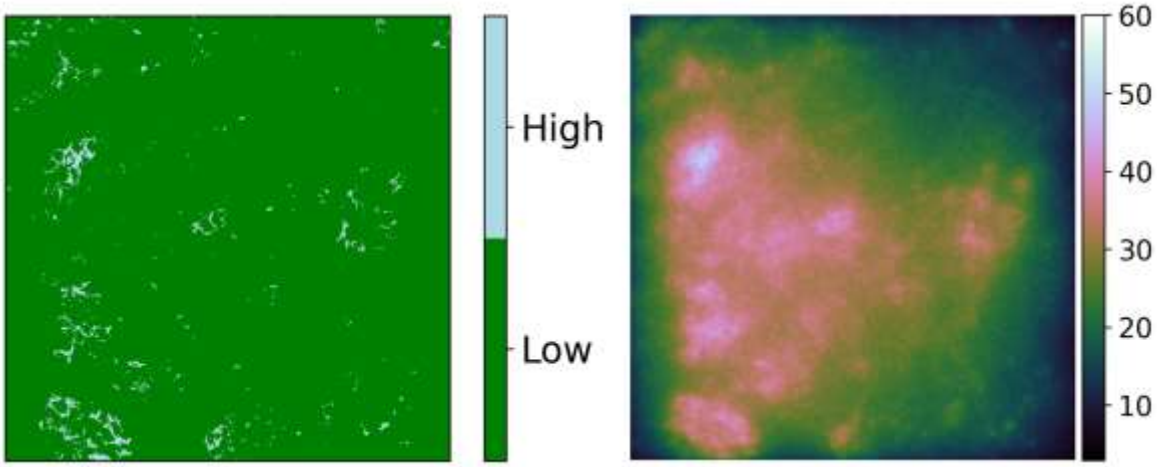
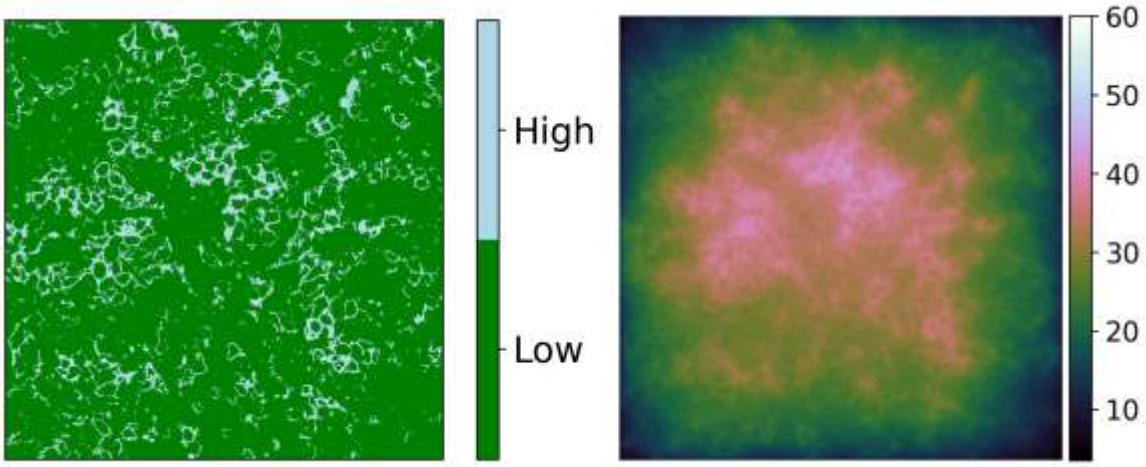
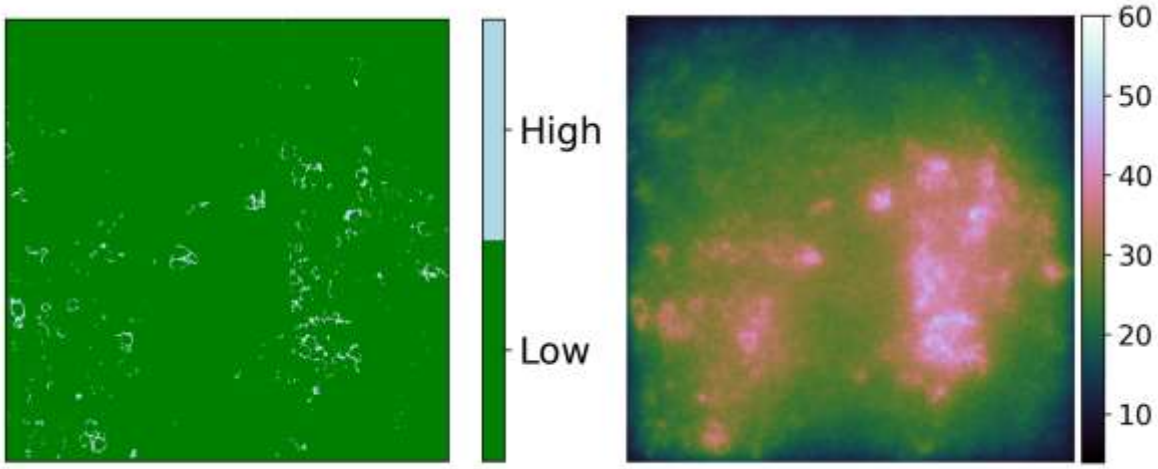


Tissue section B4 “high” SSTR2 expression

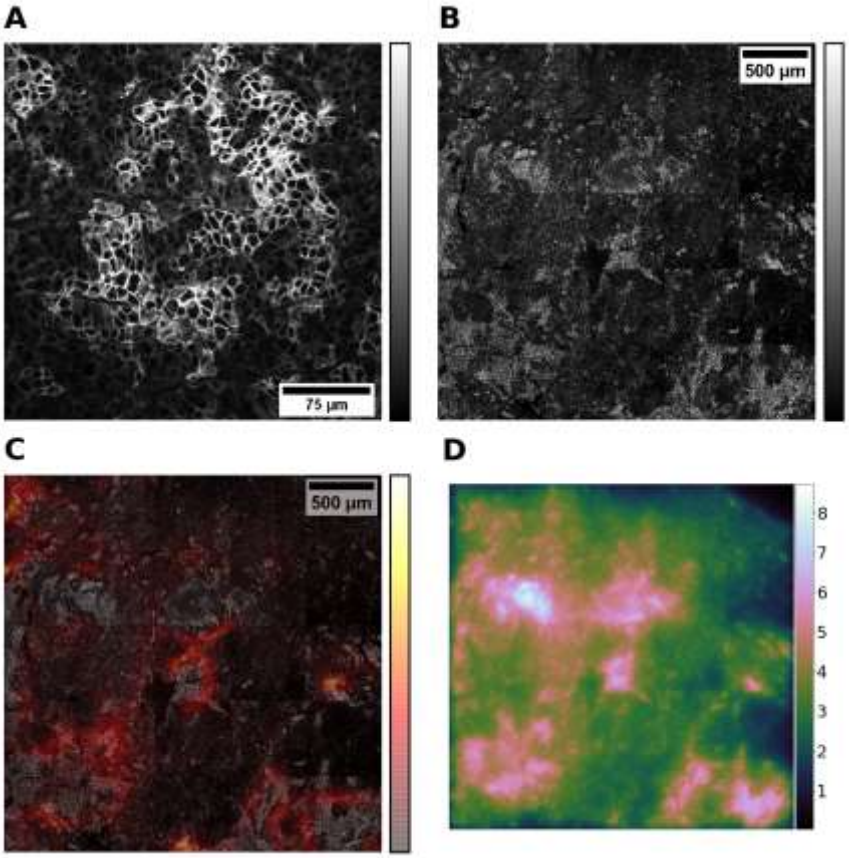


Tissue section B4 “low” SSTR2 expression

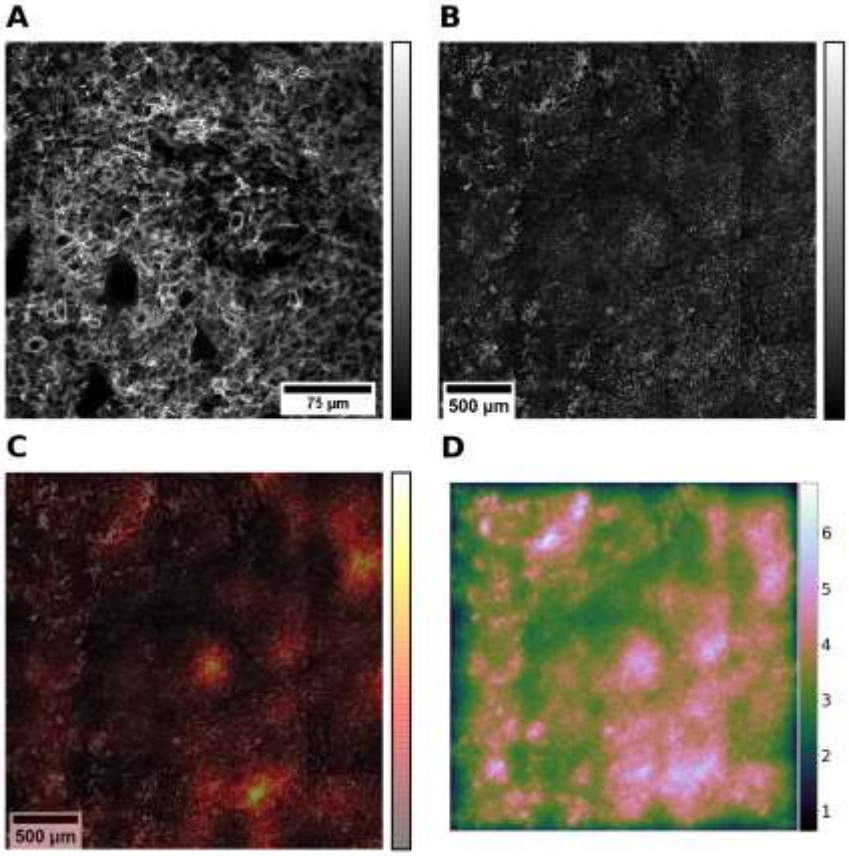


SUPPLEMENTAL FIGURE 3. Absorbed dose – DSBs correlation. Tile-scans (320 µm x 320 µm) of $SSTR_2$ stainings thresholded to identify low- (light blue) and high- (green) $SSTR_2$ expressing areas (on the left). Absorbed dose rate maps (at day 2) with color bar in Gy and mGy/h and DSBs stainings.

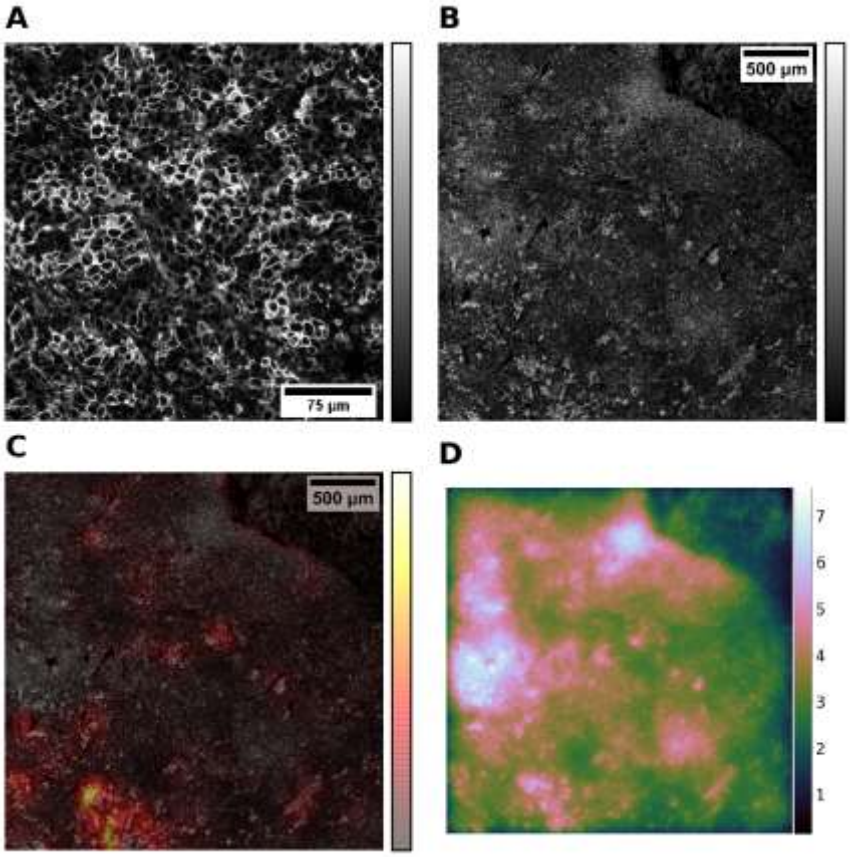
Tissue section B1 "high" $SSTR_2$ expression



Tissue section B3 "high" $SSTR_2$ expression

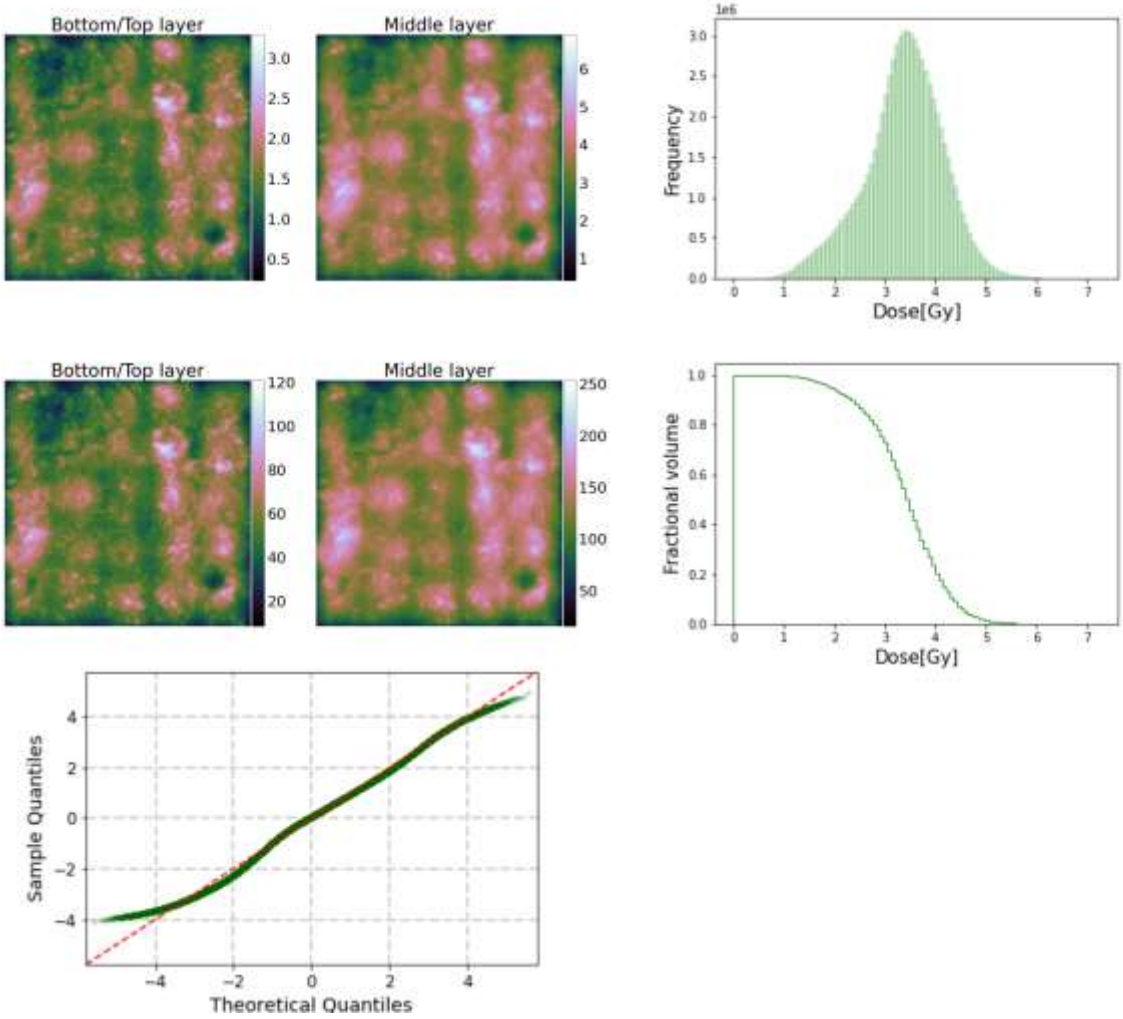


Tissue section B4 "high" $SSTR_2$ expression

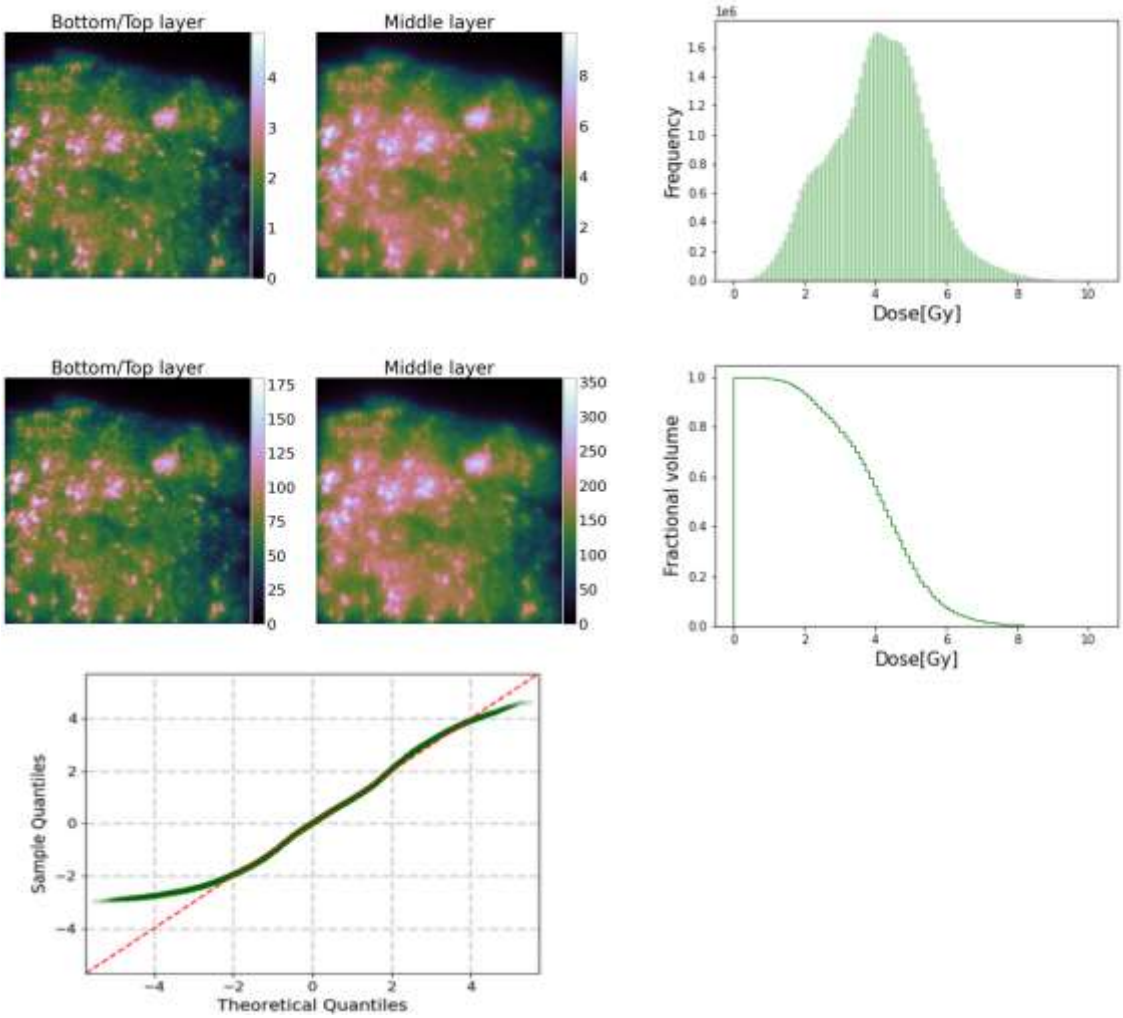


SUPPLEMENTAL FIGURE 4. Template matching technique. (A) Small tissue section used as template. (B) Large tissue section used as "test image". (C) Color map indicating the similarity score based on the χ^2 value overlaid on top of the large tissue section. Color bars indicate the pixel intensities of the tile-scans (greyscale) or similarity map (red-yellow). (D) Absorbed dose map with color bar in Gy.

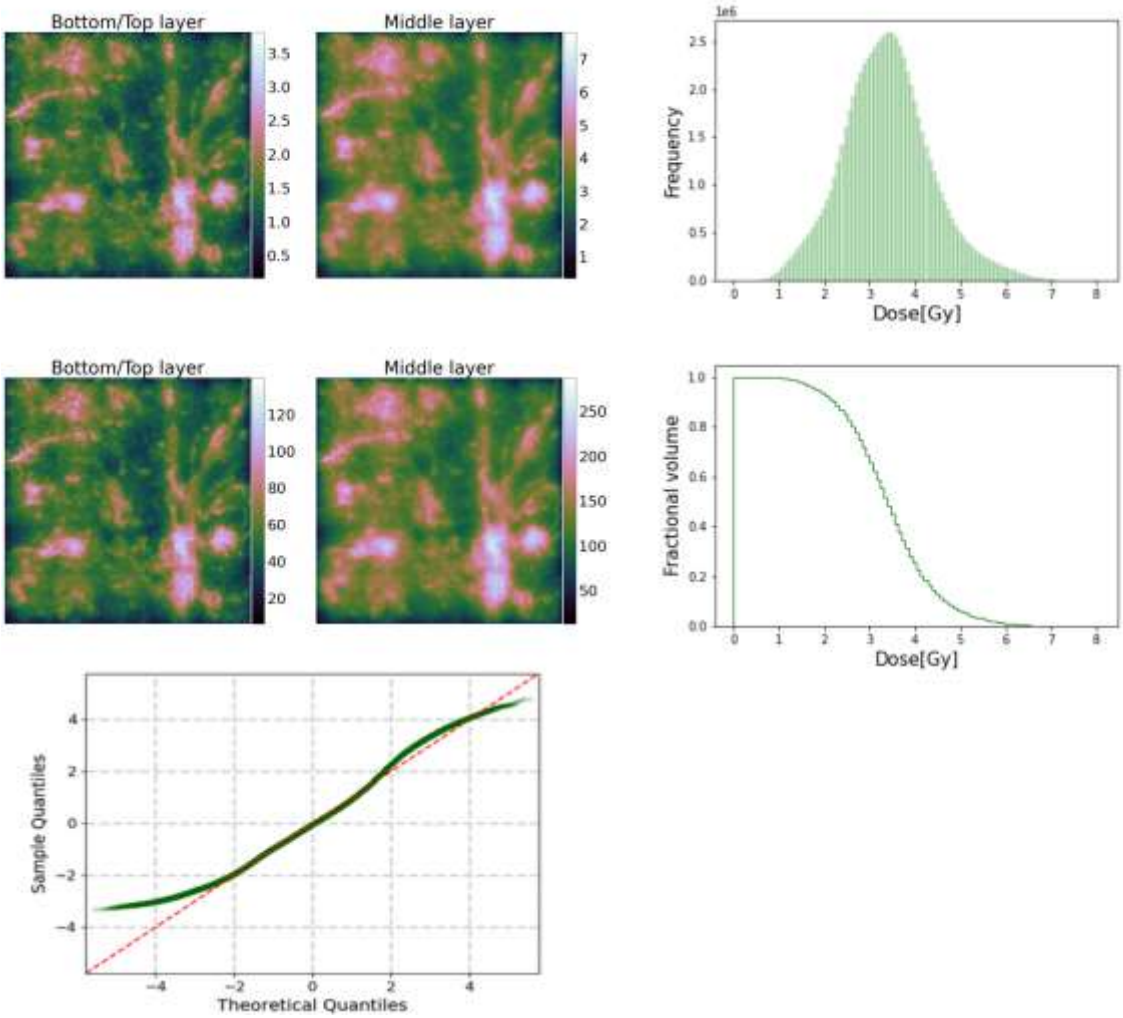
Absorbed dose 0-2days and initial dose-rate
Tissue section A1



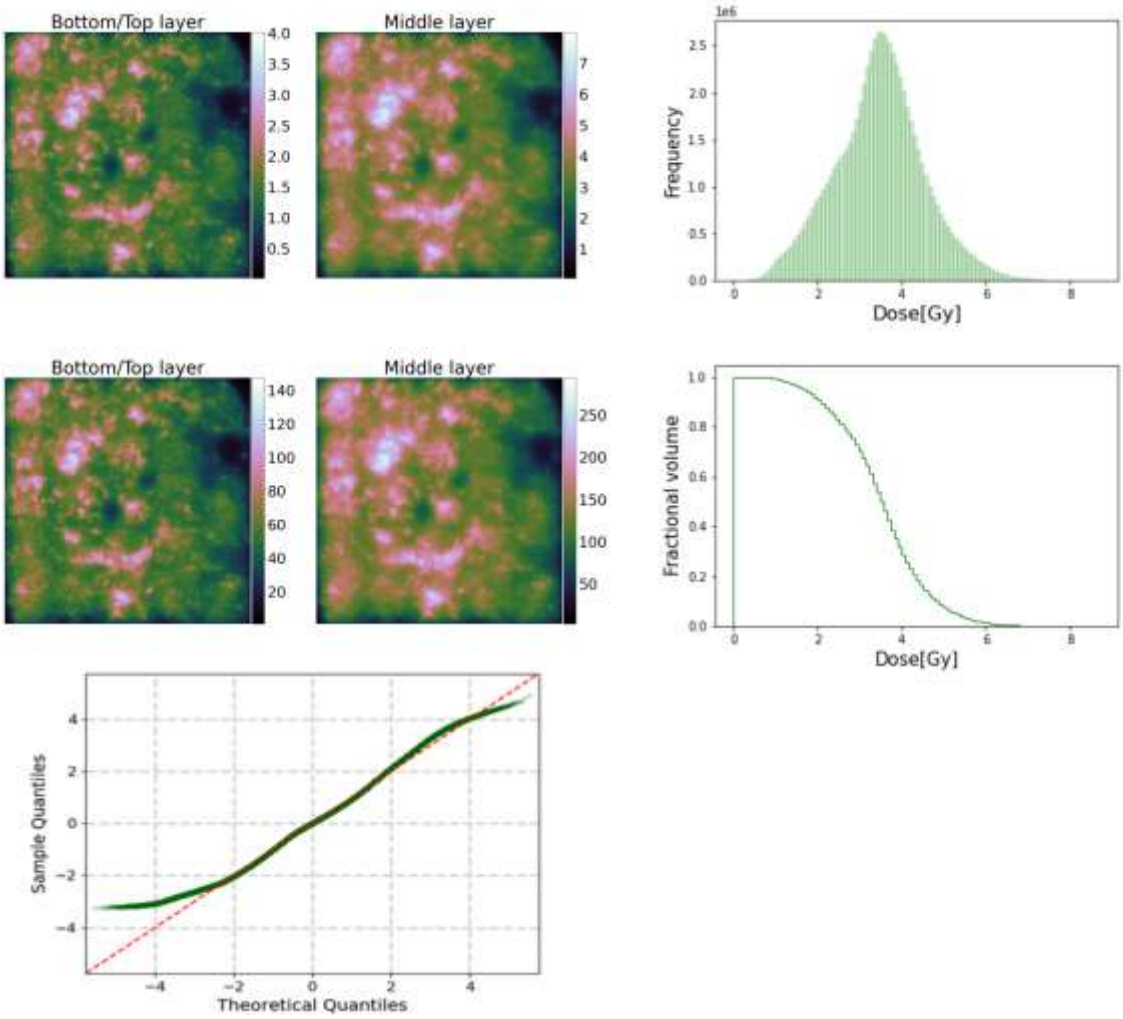
Tissue section A2



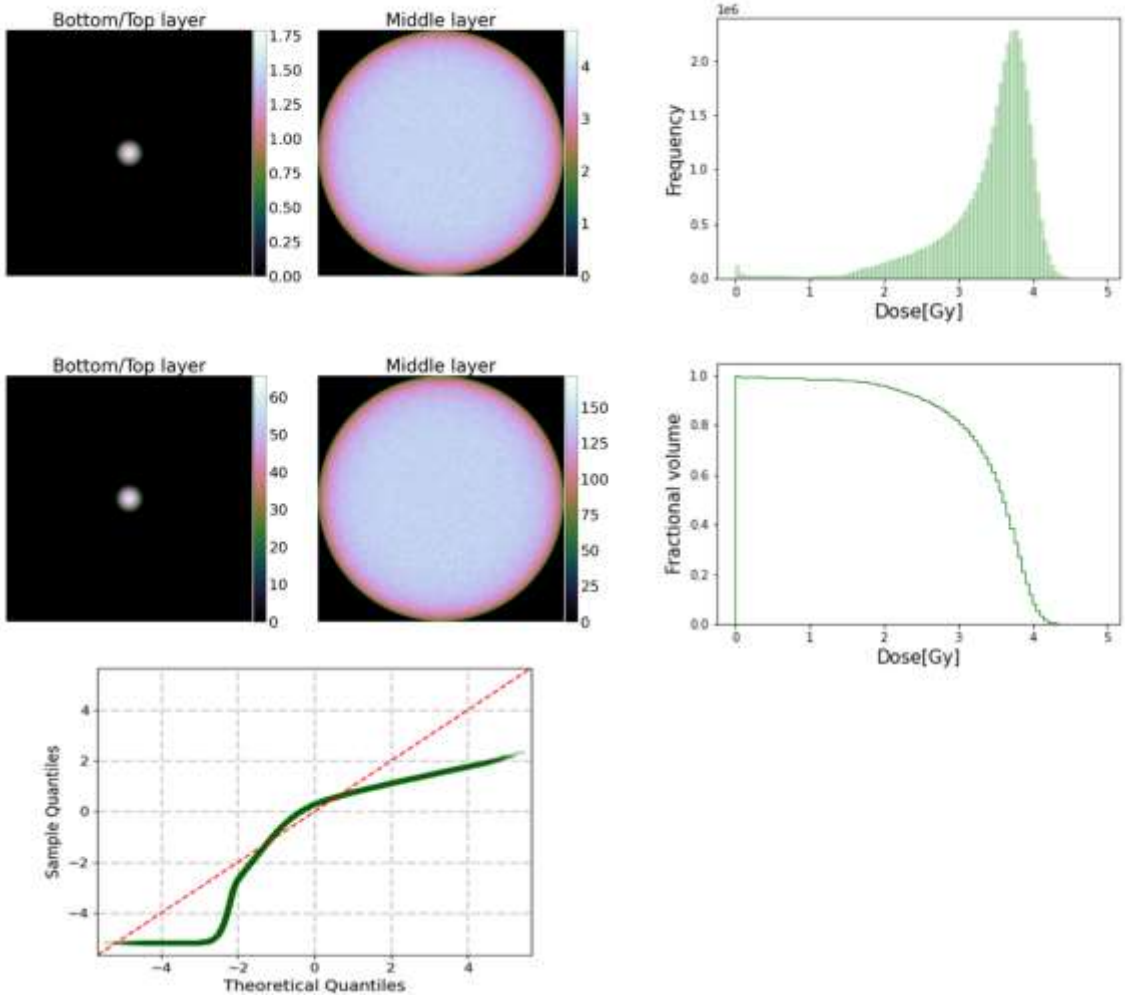
Tissue section A3



Tissue section A4

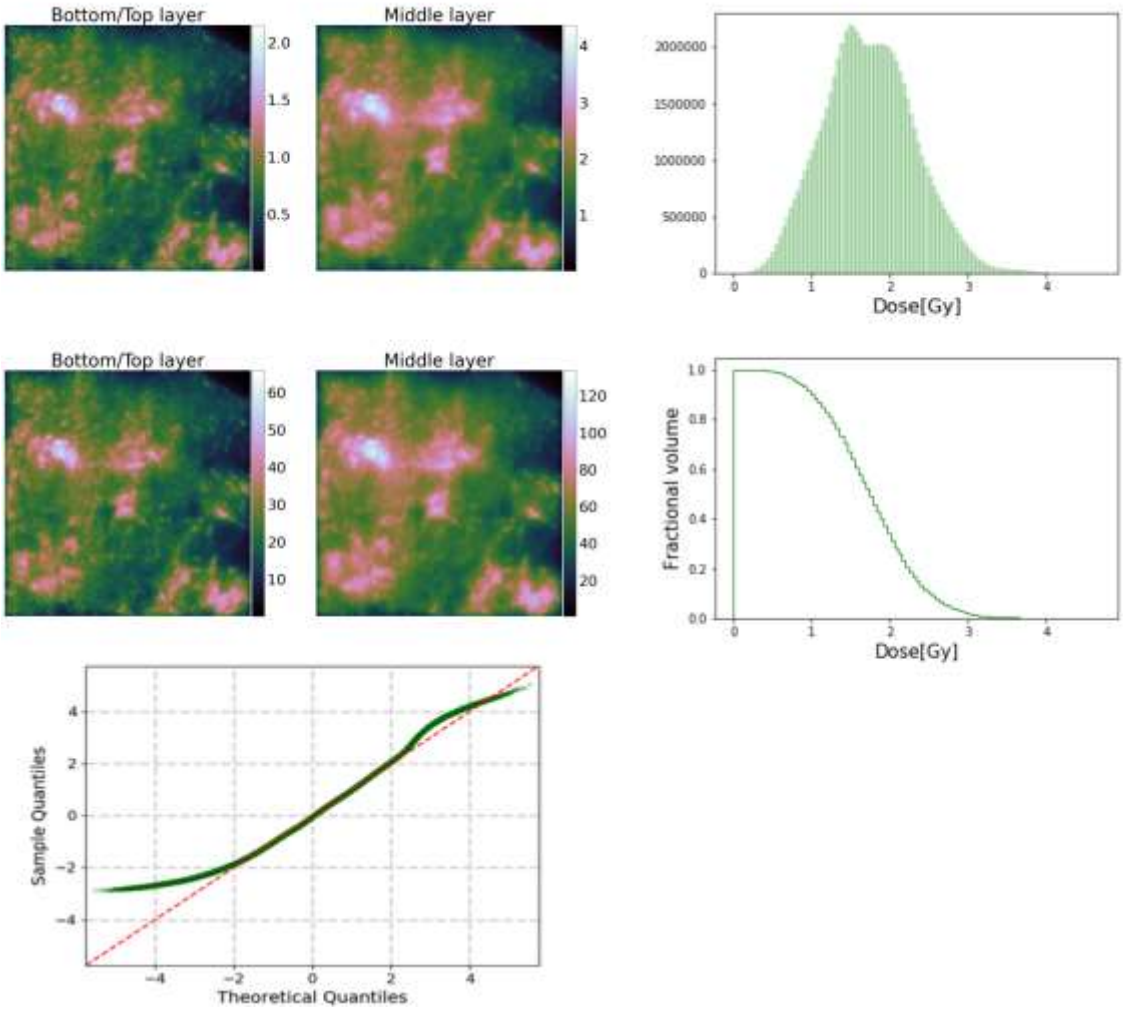


Equivalent uniform case

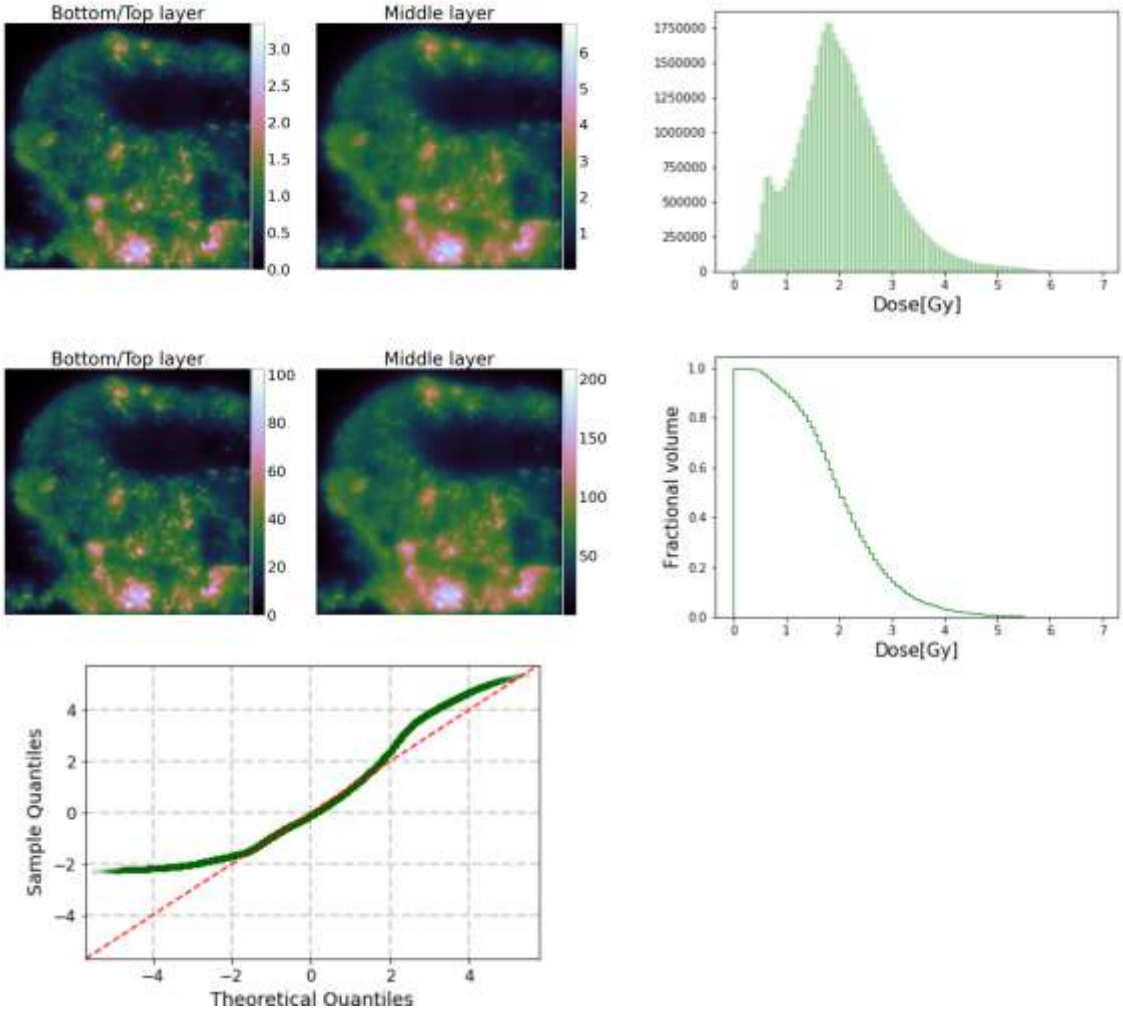


Absorbed dose 2-5 days and dose-rate at day 2

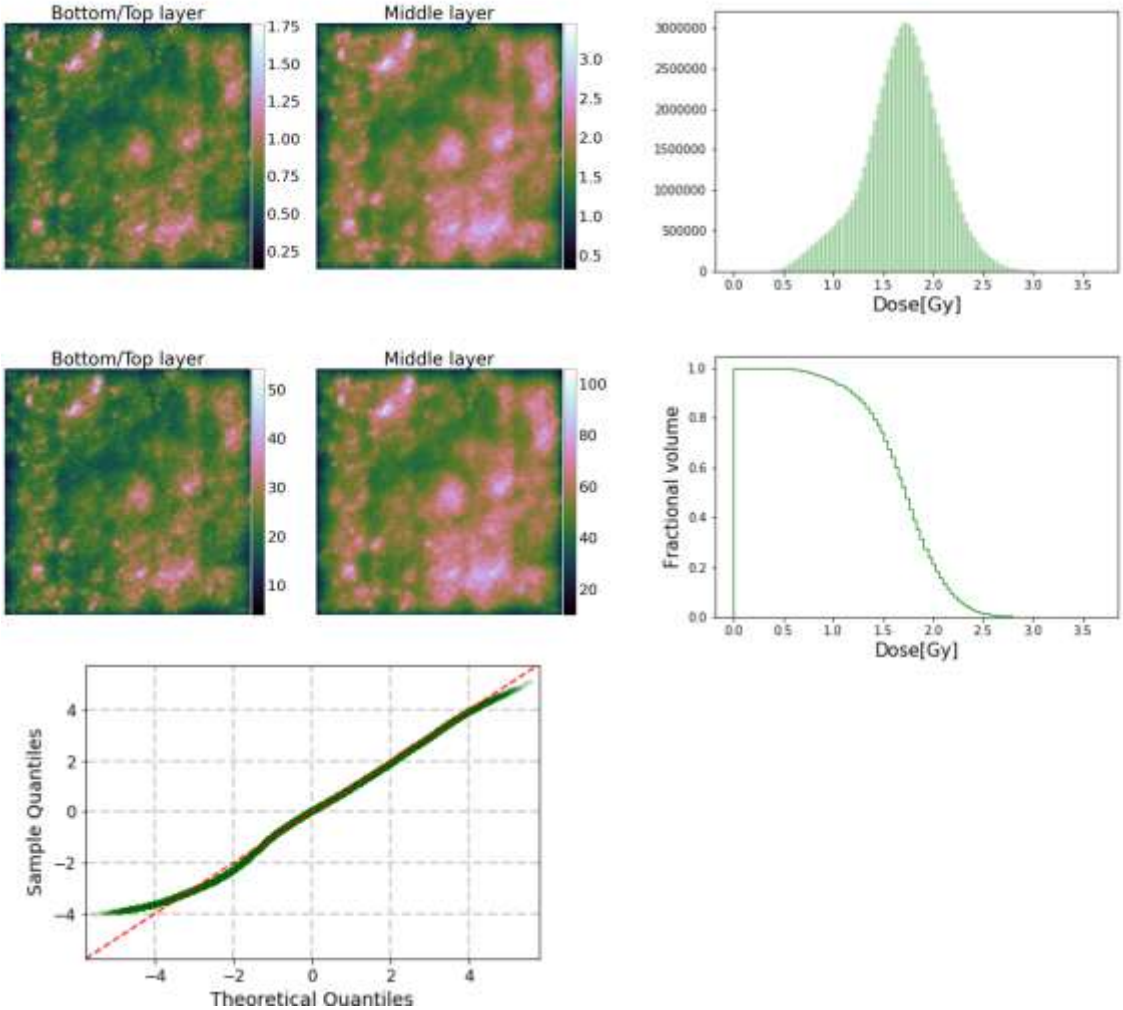
Tissue section B1



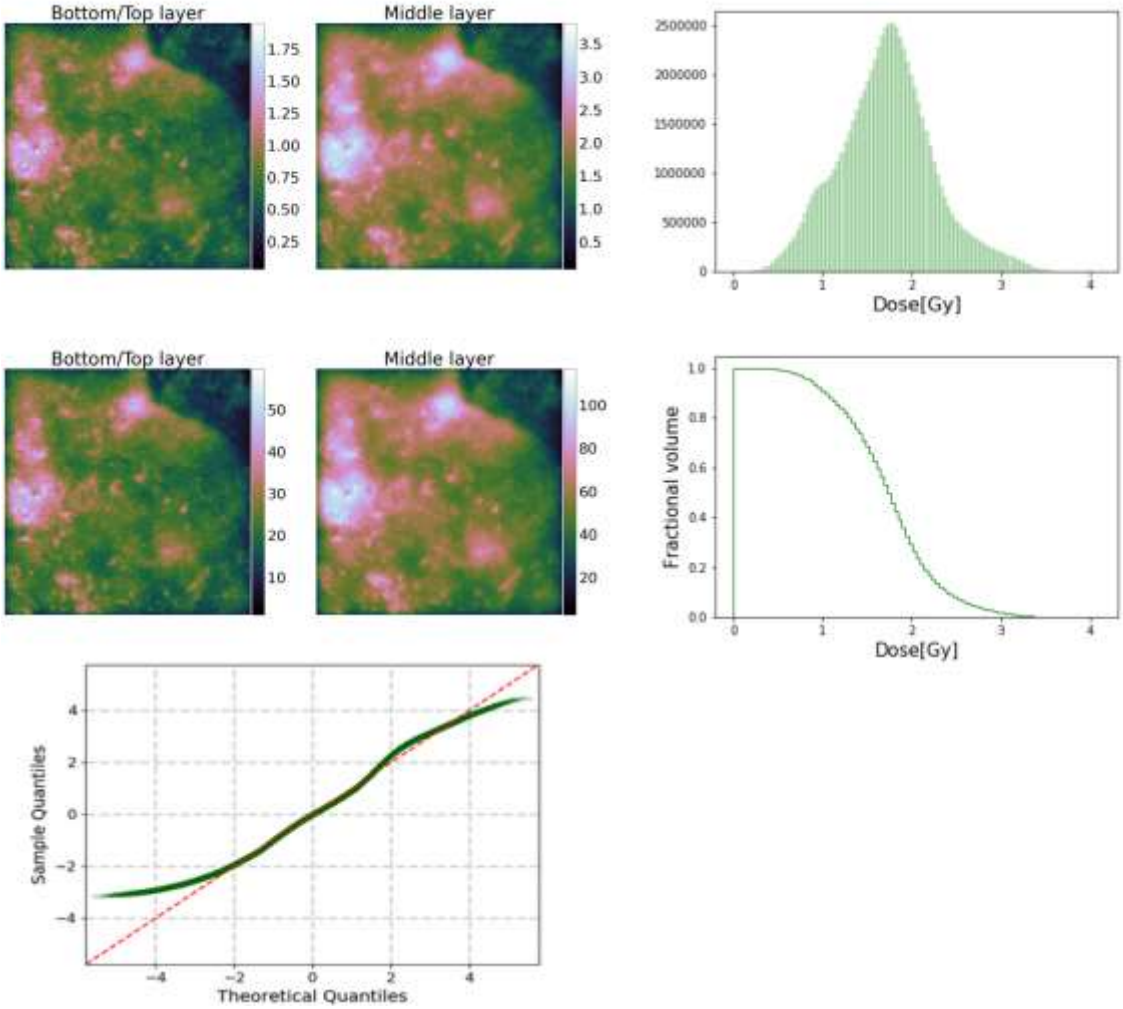
Tissue section B2



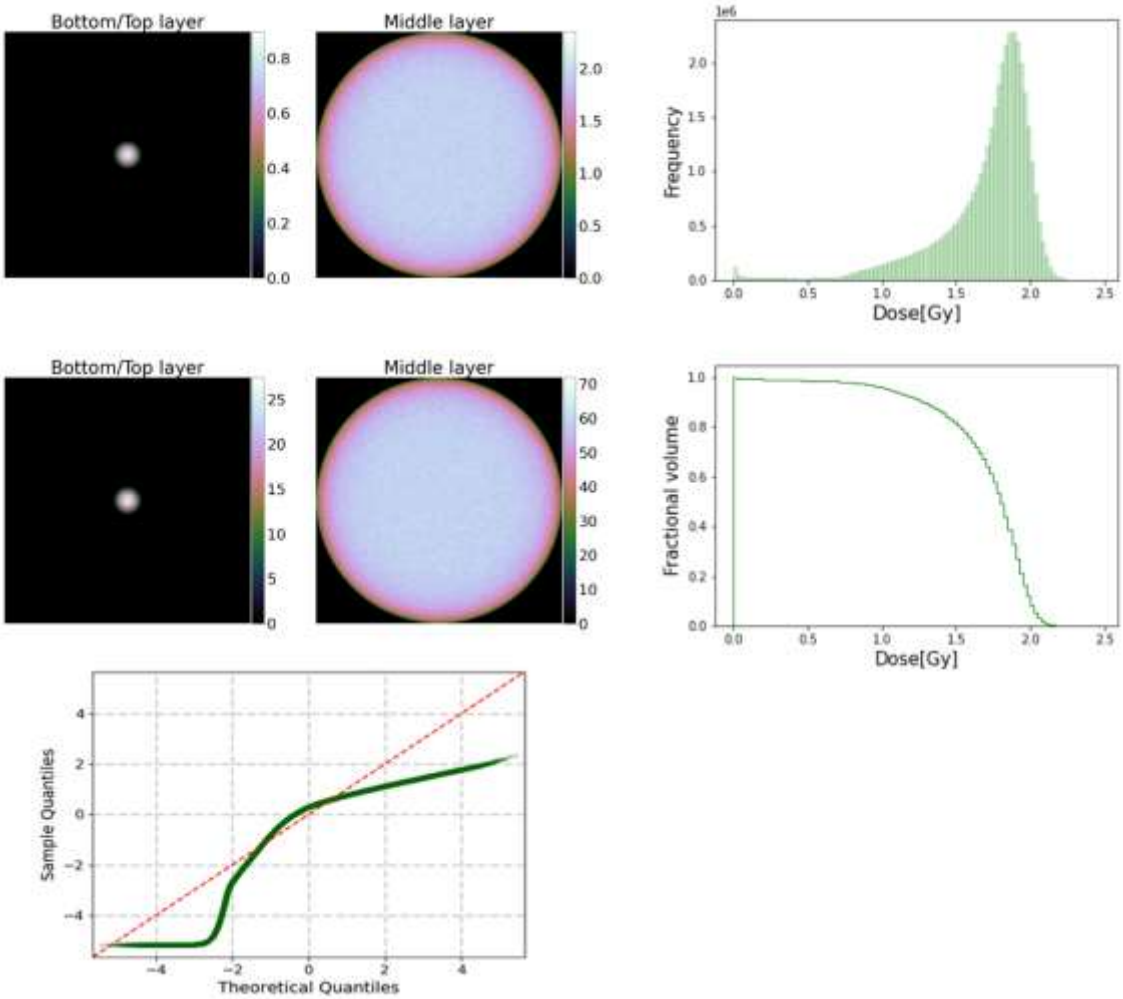
Tissue section B3



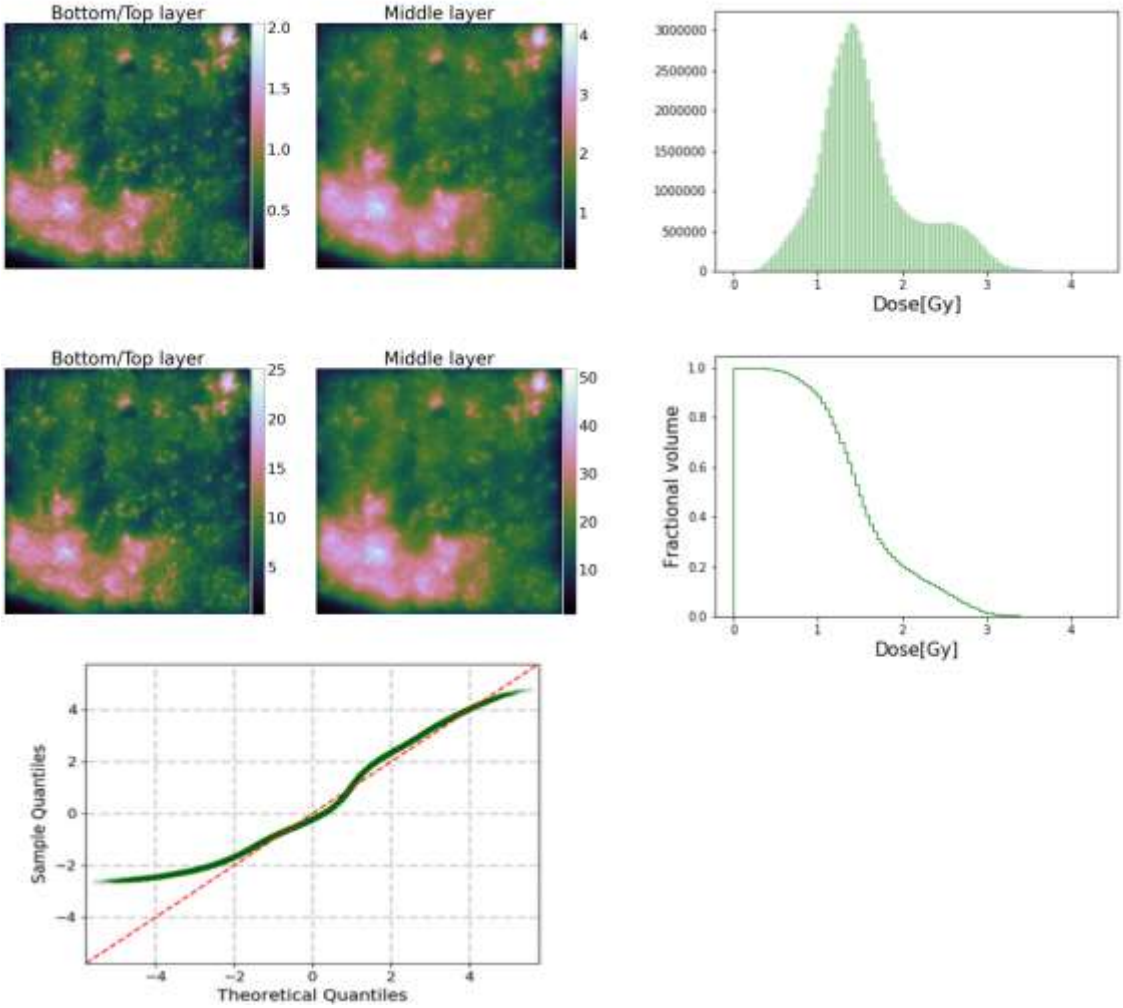
Tissue section B4



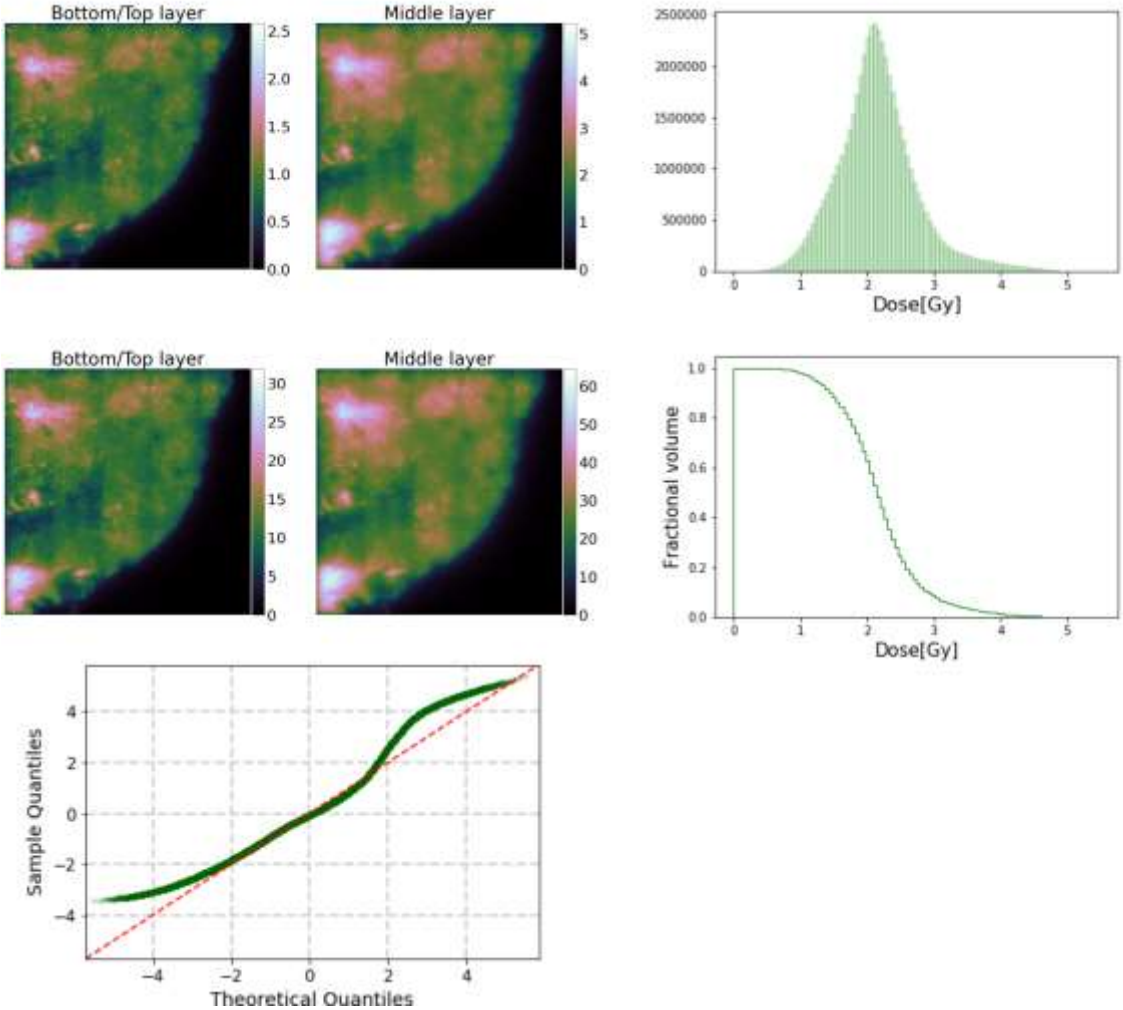
Equivalent uniform case



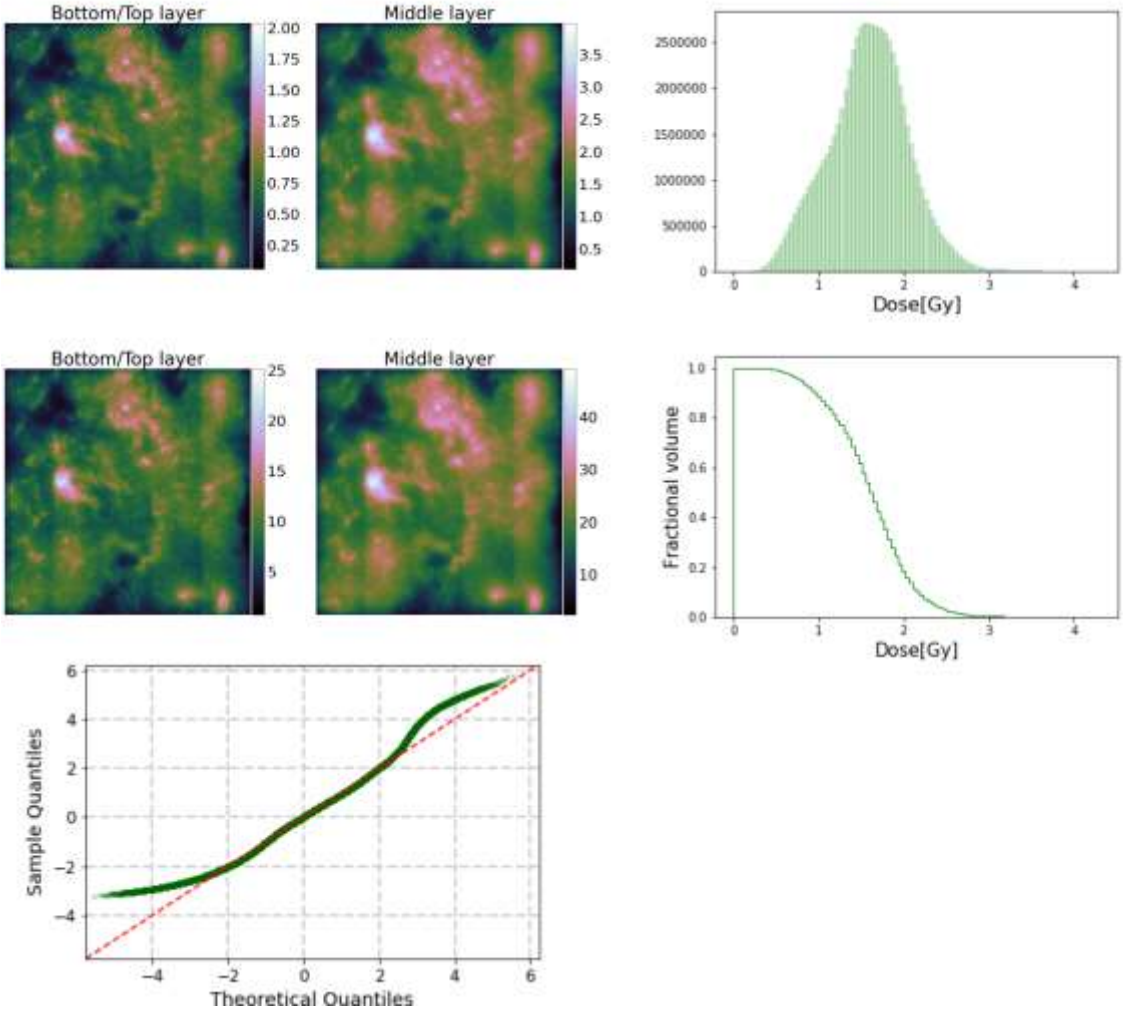
Absorbed dose 5-11 days and dose-rate at day 5
Tissue section C1



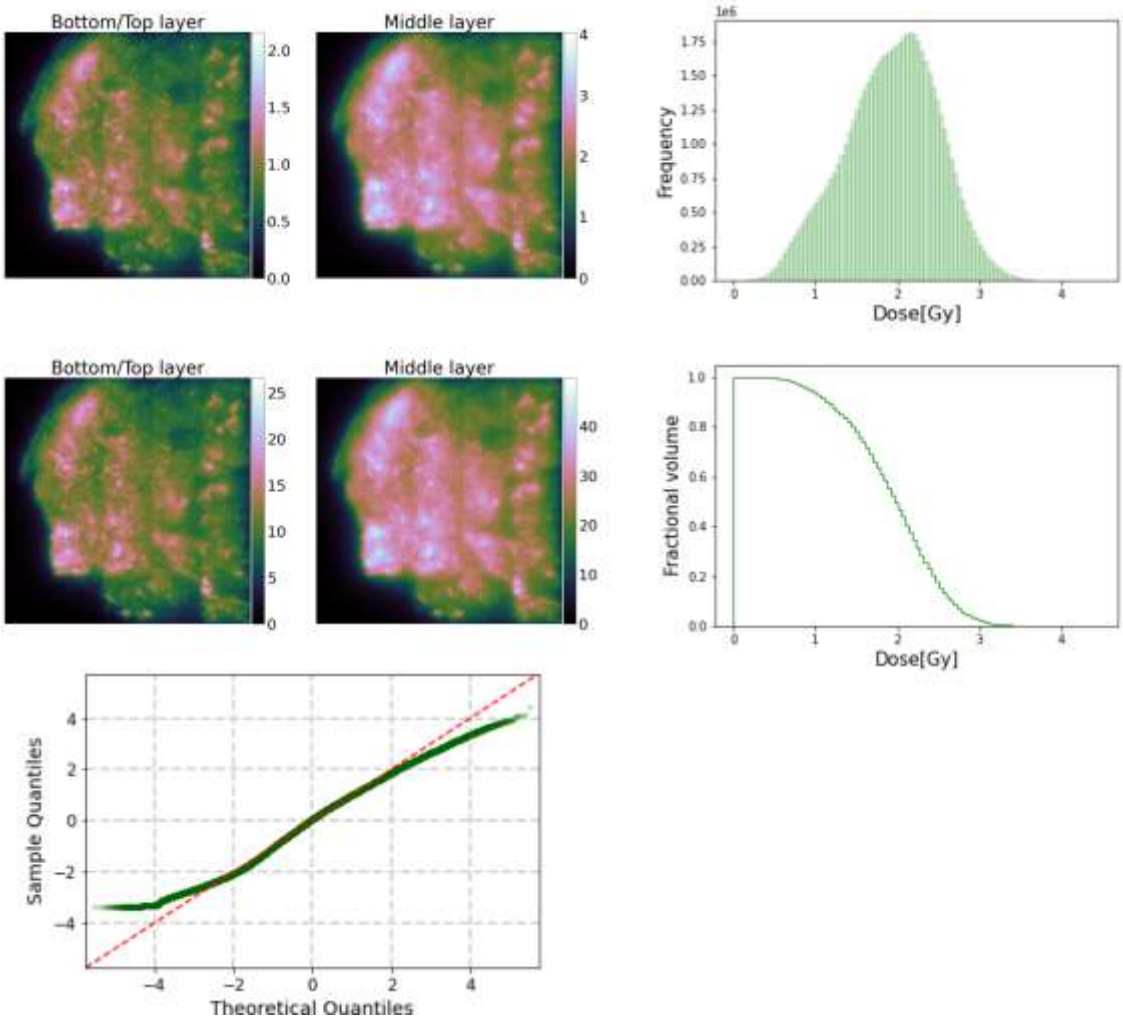
Tissue section C2



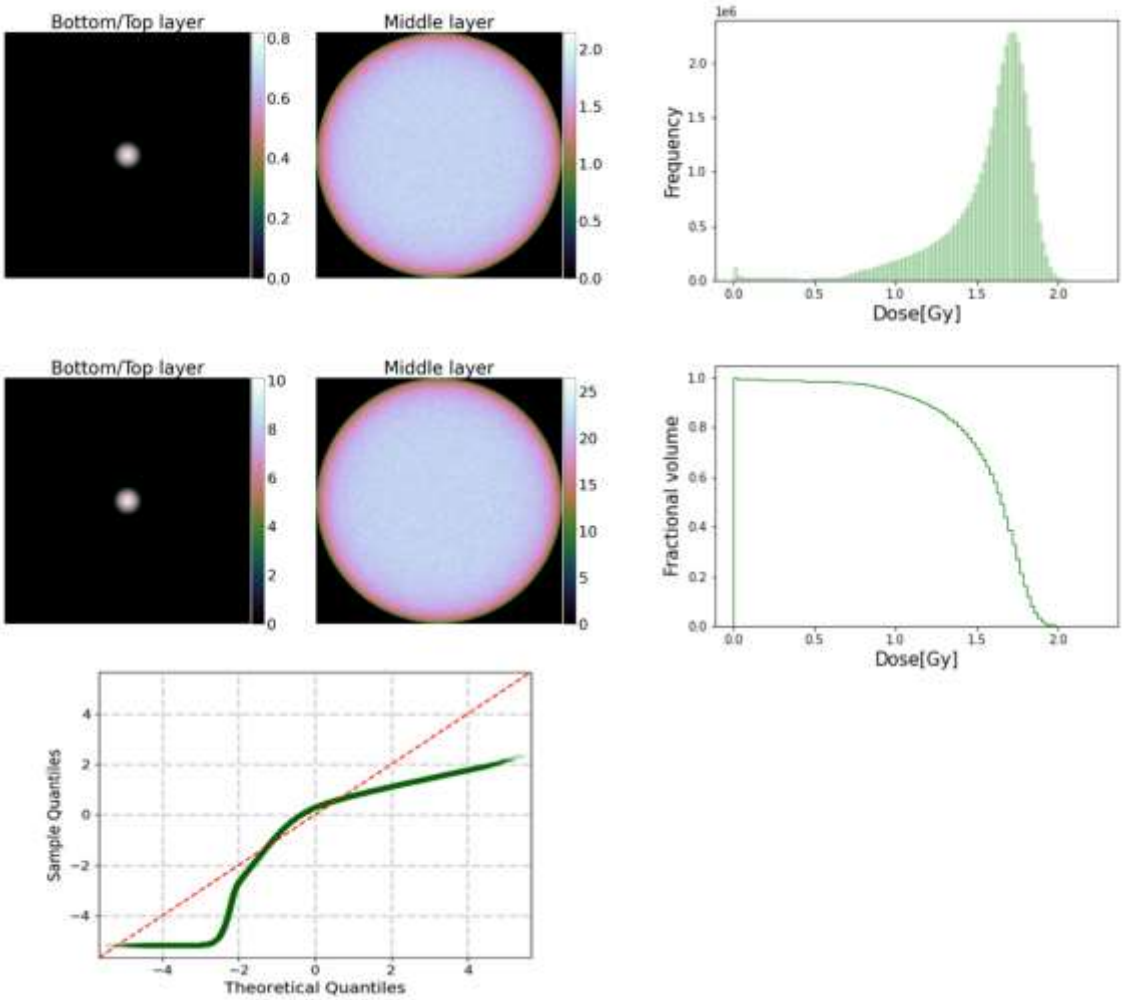
Tissue section C3



Tissue section C4

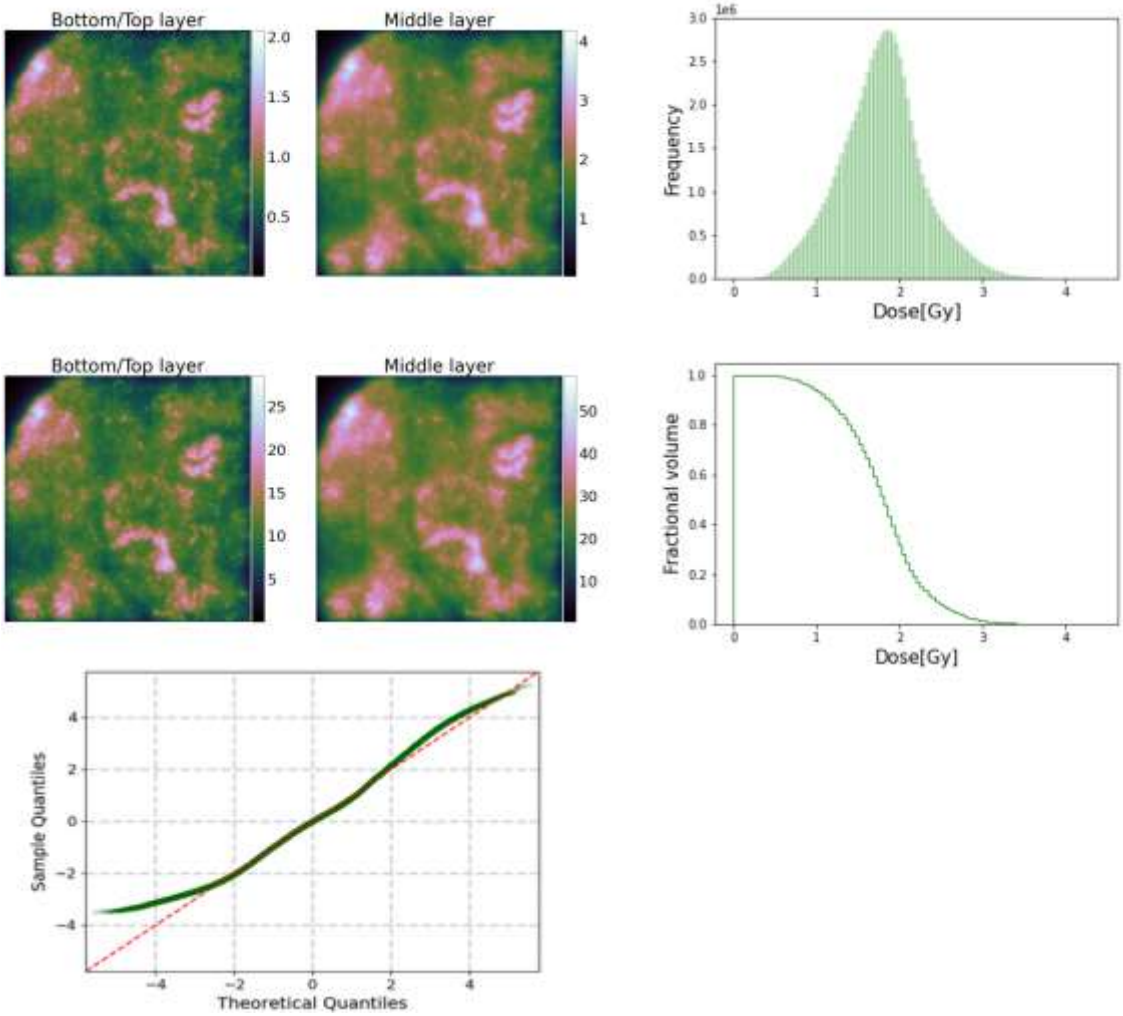


Equivalent uniform case

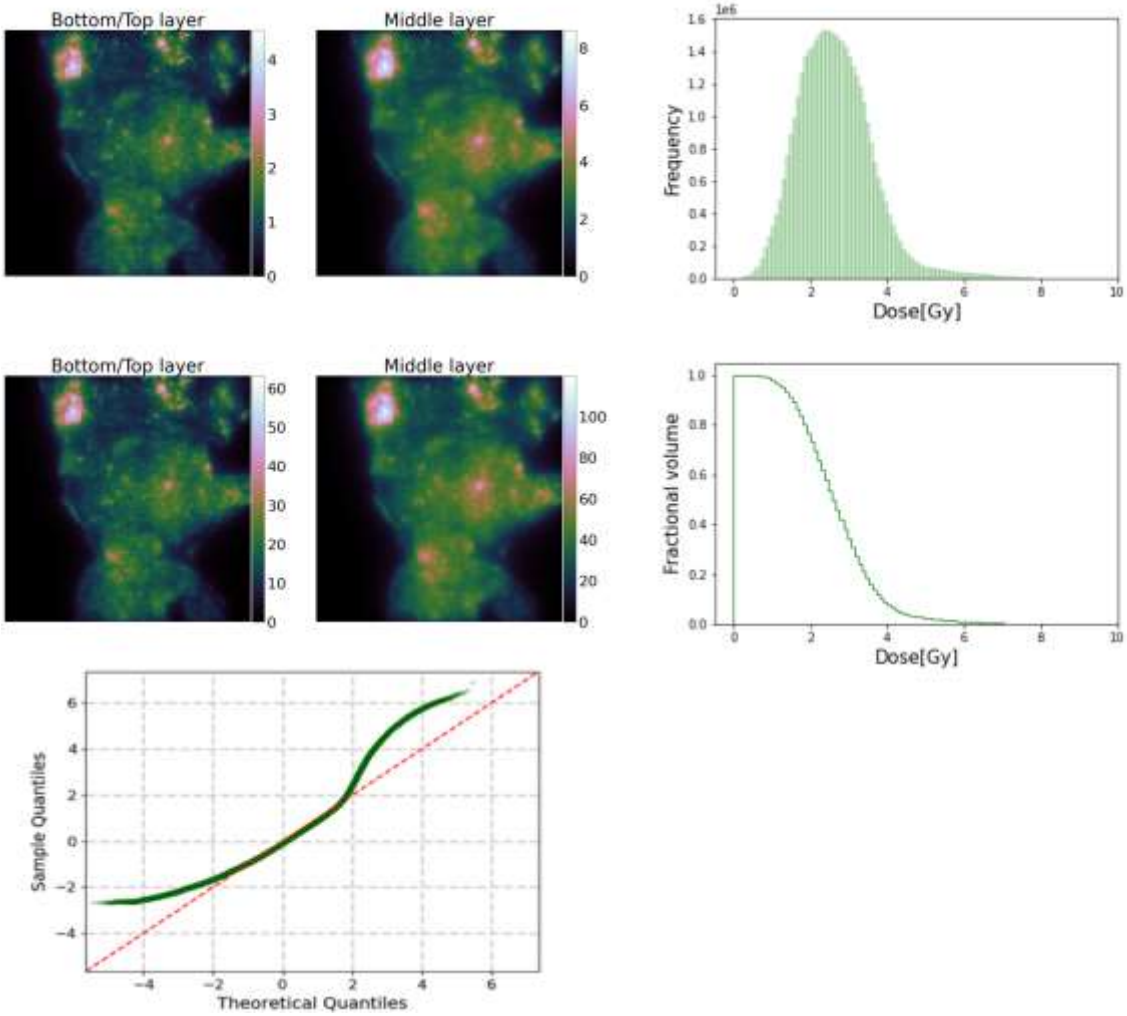


Absorbed dose 11-14 days and dose-rate at day 11

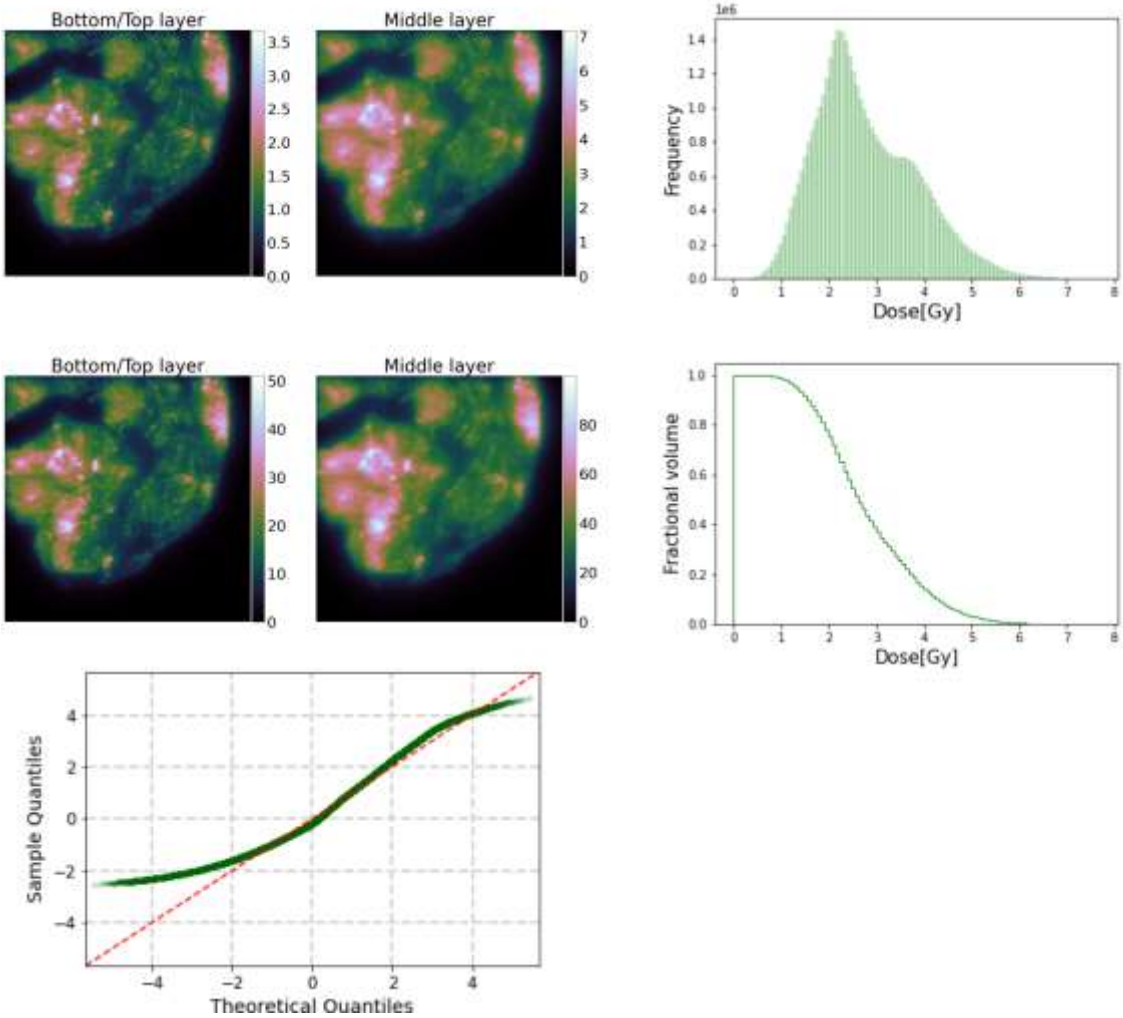
Tissue section D1



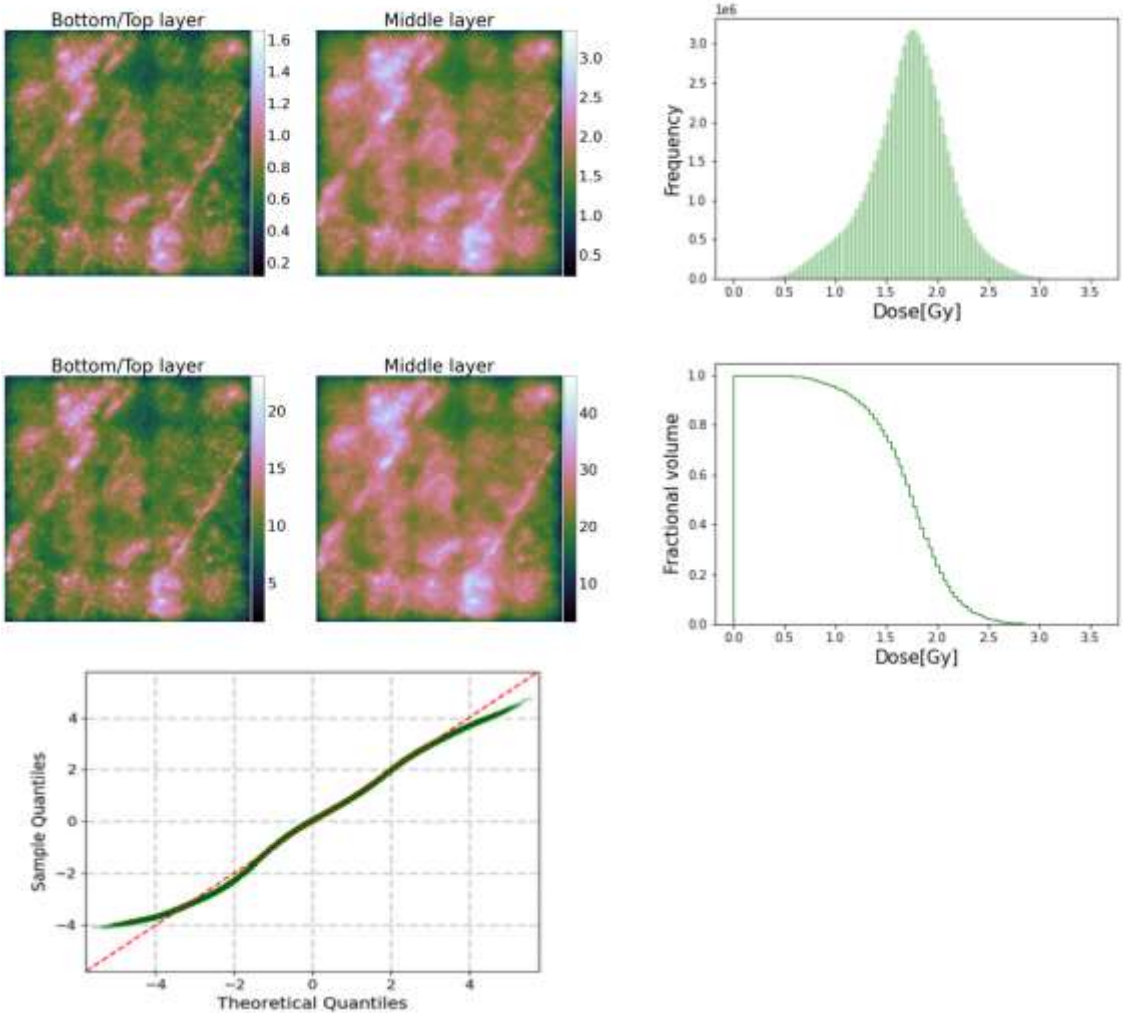
Tissue section D2



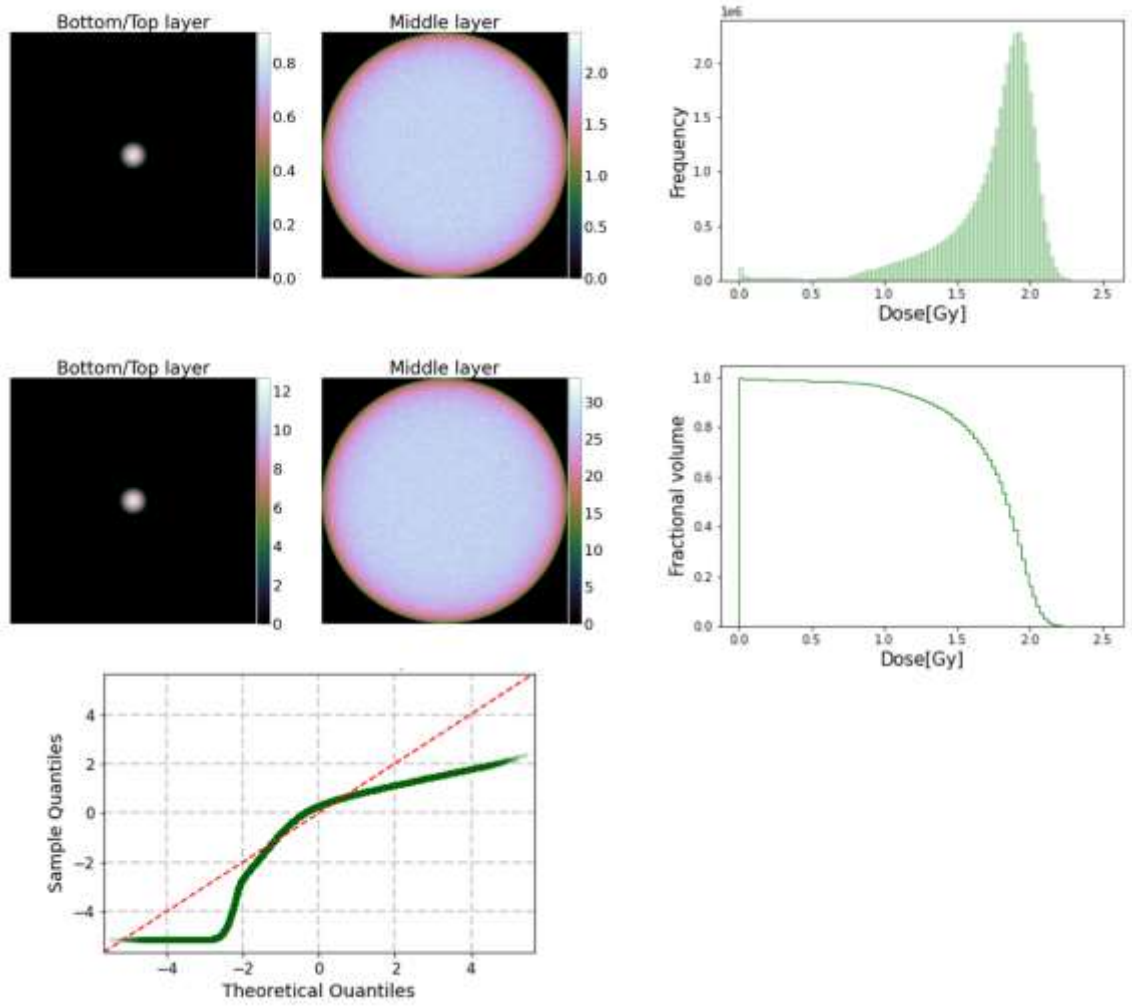
Tissue section D3



Tissue section D4

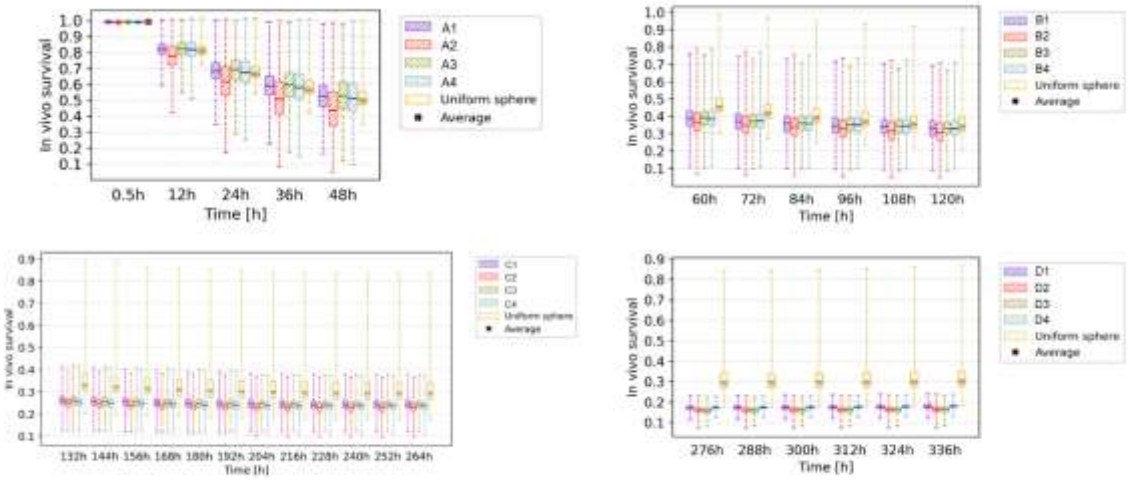


Equivalent uniform case

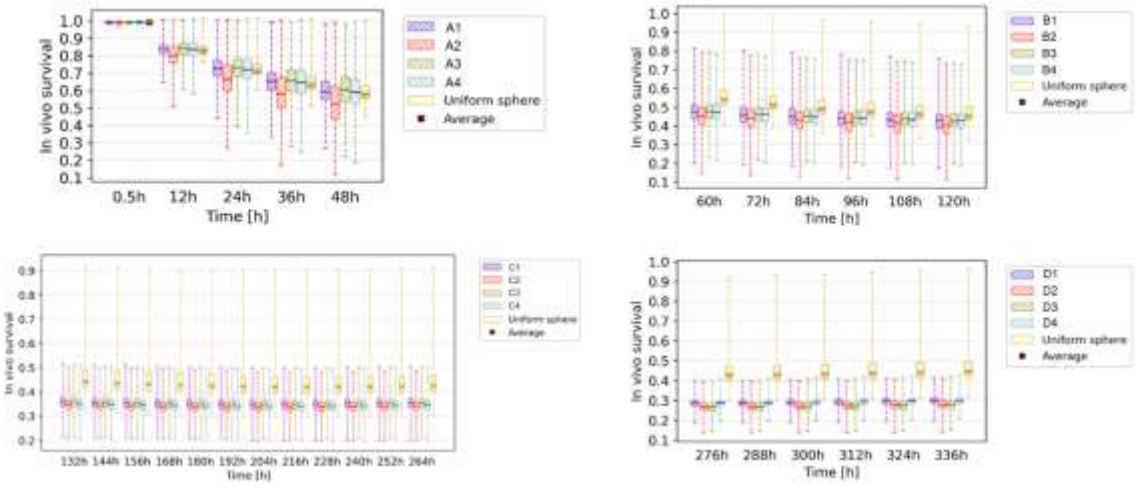


SUPPLEMENTAL FIGURE 5. Absorbed dose distributions corresponding to homogeneous and heterogeneous exposures reported by means of dose and dose rate maps (left side), frequency DVH and cumulative DVH (right side)and Q-Q plots (bottom). The generalized equivalent uniform dose (gEUD) for each tissue section is reported in Supplemental Table 2.

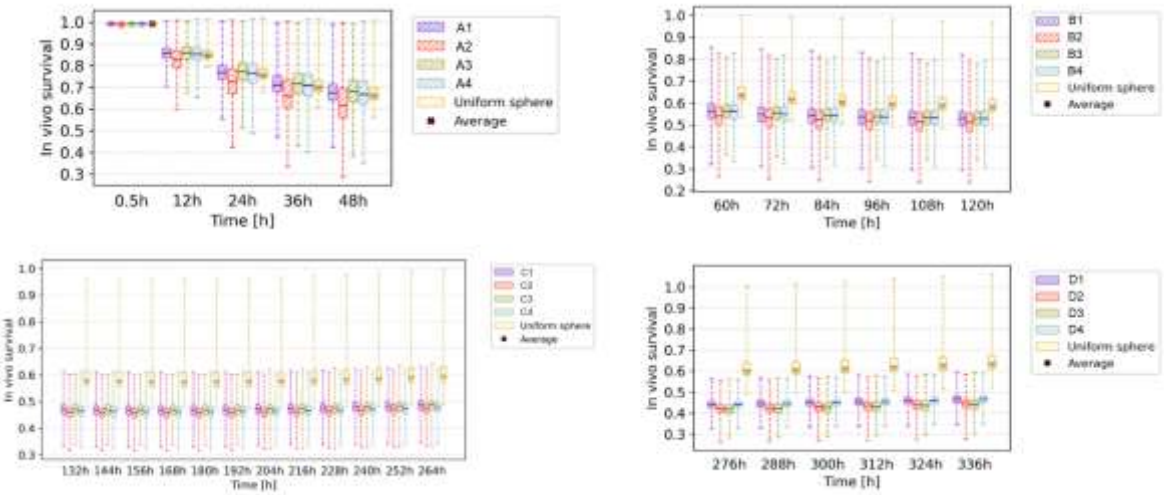
Parameters α=0.14 Gy^{-1}, α/β=5 Gy



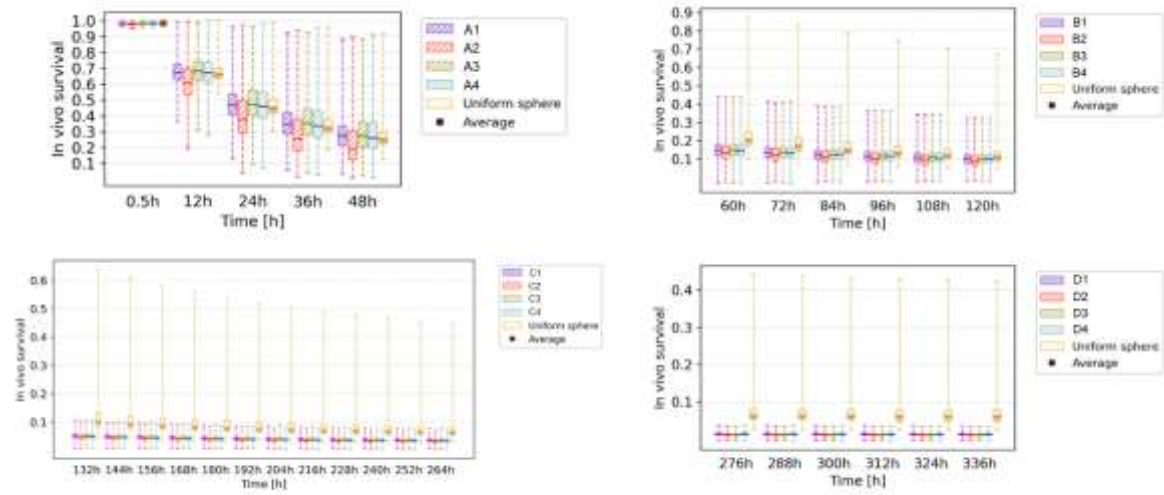
Parameters α=0.14 Gy^{-1}, α/β=10 Gy



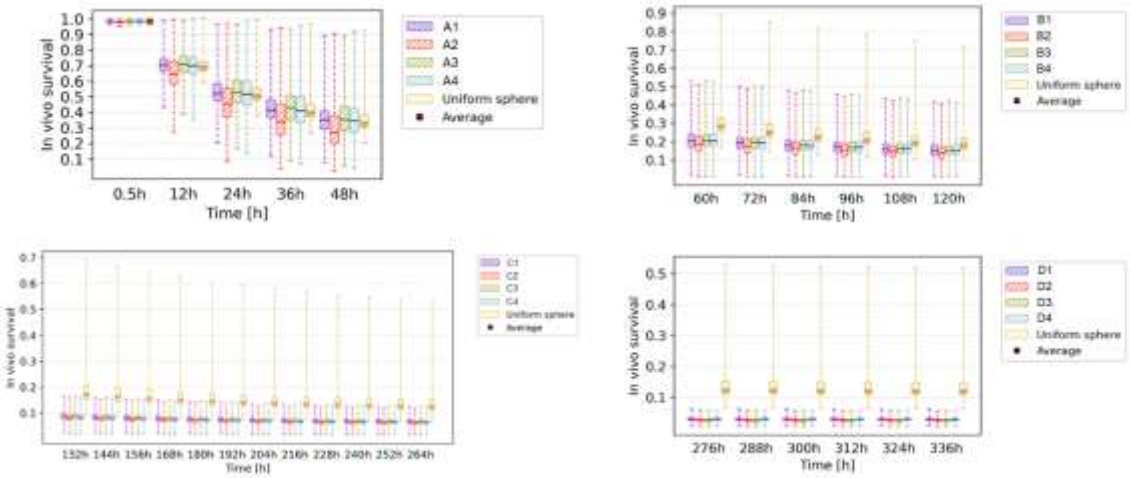
Parameters α=0.14 Gy^{-1}, α/β=100 Gy



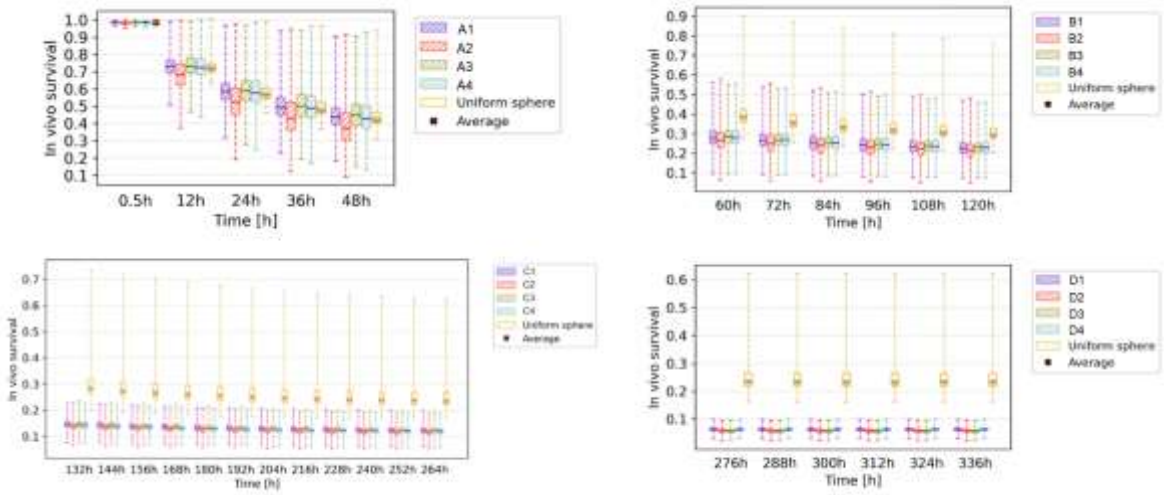
Parameters α=0.264 Gy^{-1}, α/β=5 Gy



Parameters α=0.264 Gy^{-1}, α/β=10 Gy

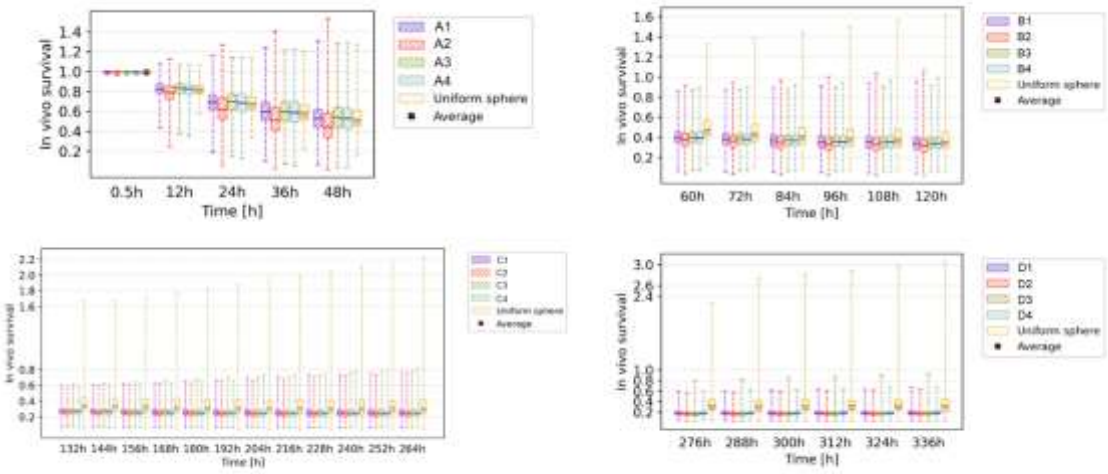


Parameters α=0.264 Gy^{-1}, α/β=100 Gy

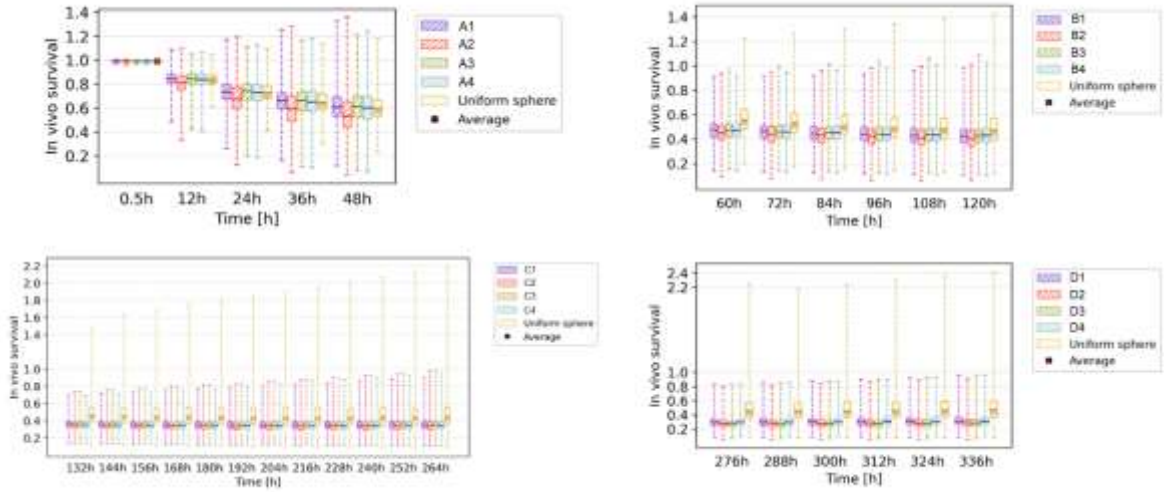


SUPPLEMENTAL FIGURE 6. Box plots indicating the *in vivo* survival distribution over time for constant values of α and β on different excised tissue sections. The whiskers correspond to 1.5 times the interquartile range.

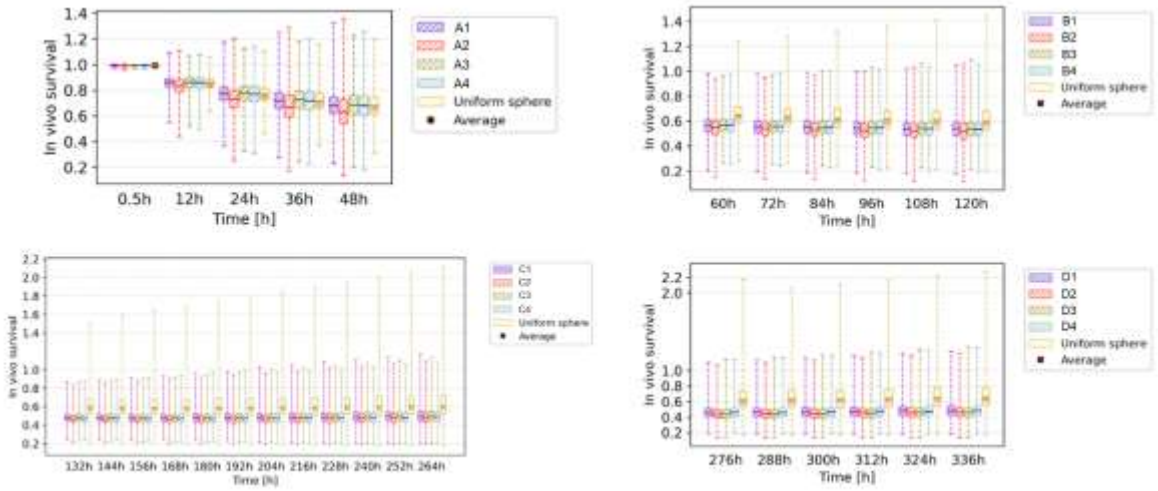
Parameters α=(0.14±0.03) Gy^{-1}, α/β=5 Gy



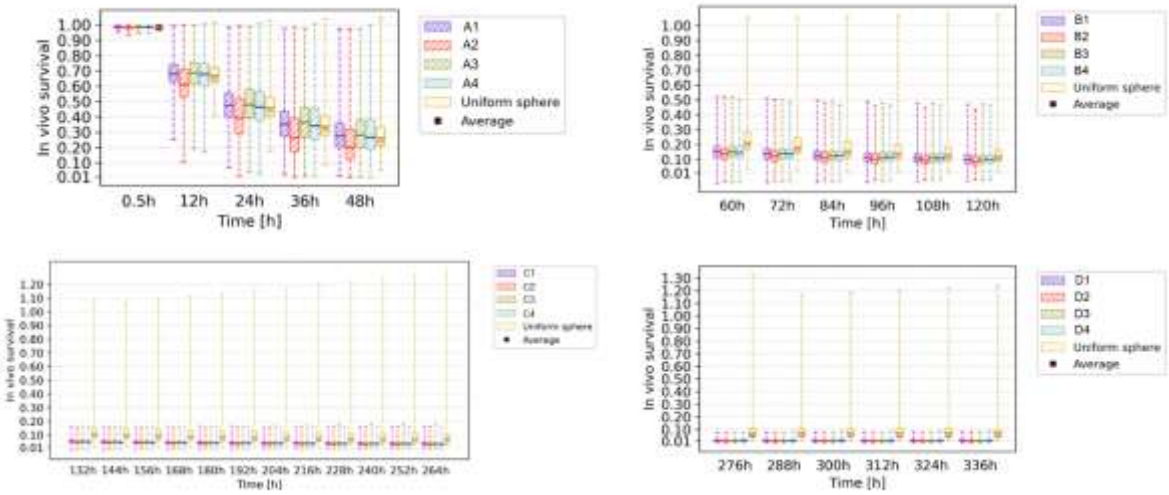
Parameters α=(0.14±0.03) Gy^{-1}, α/β=10 Gy



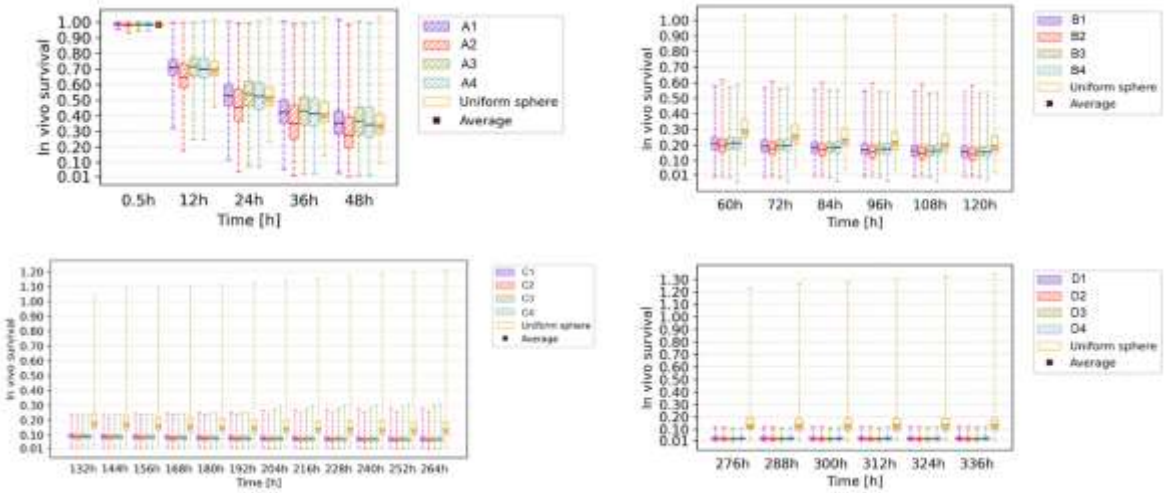
Parameters α=(0.14±0.03) Gy^{-1}, α/β=100 Gy



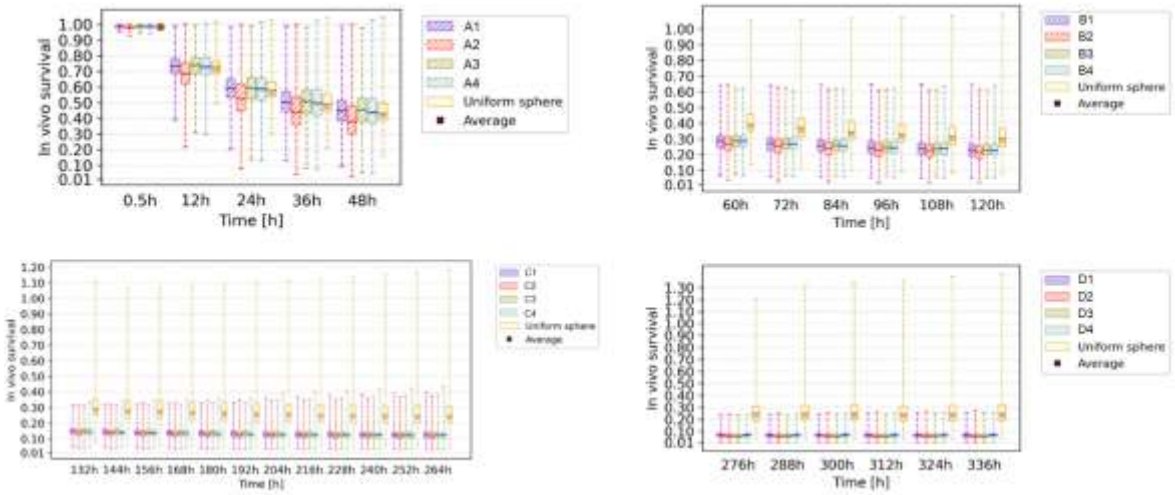
Parameters α=(0.264±0.04) Gy^{-1}, α/β=5 Gy



Parameters α=(0.264±0.04) Gy^{-1}, α/β=10 Gy

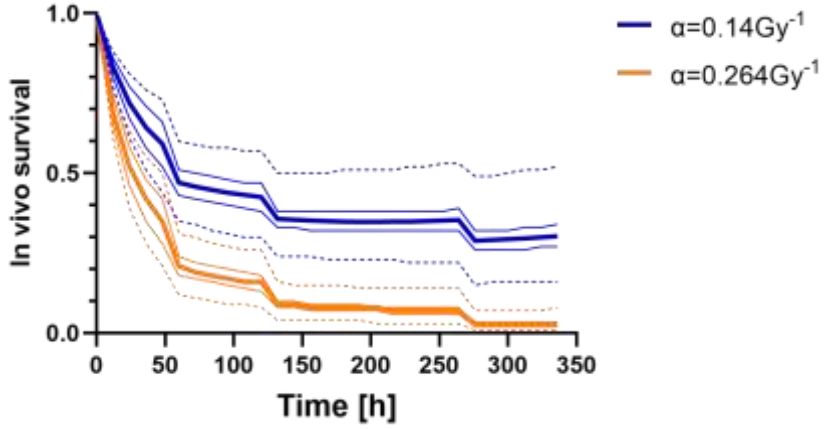


Parameters α=(0.264±0.04) Gy^{-1}, α/β=100 Gy

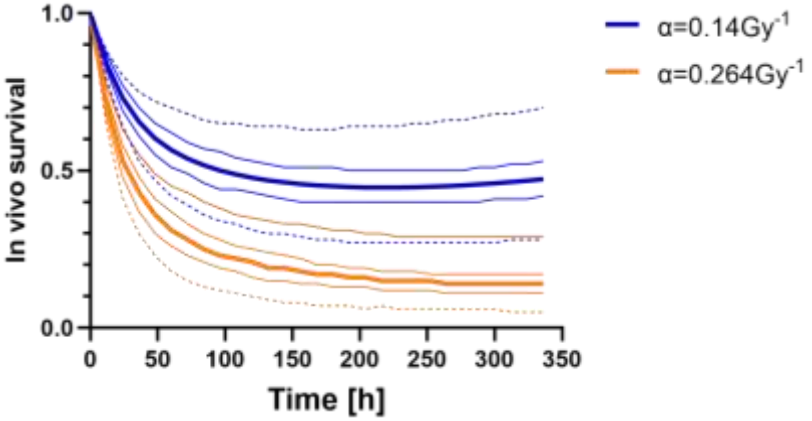


SUPPLEMENTAL FIGURE 7. Box plots indicating the *in vivo* survival distribution over time for Gaussian distributed α and β values on different excised tissue sections. The whiskers correspond to 1.5 times the interquartile range.

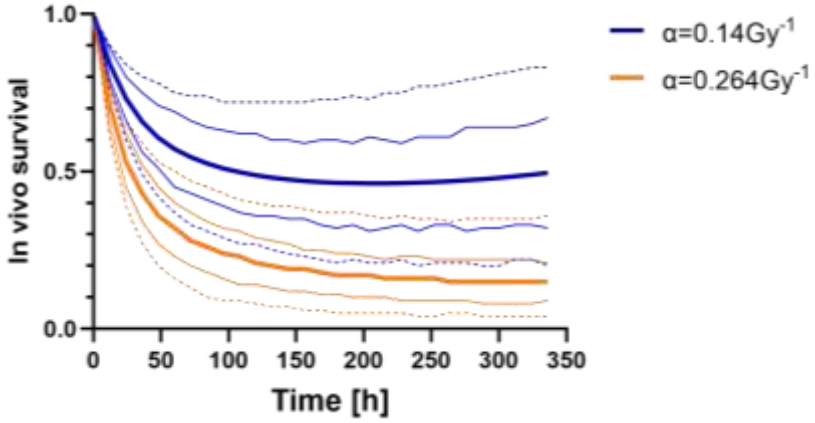
Heterogeneous dose distribution - Gaussian sensitivity



Uniform dose distribution - constant sensitivity



Uniform dose distribution - Gaussian sensitivity



SUPPLEMENTAL FIGURE 8. Radiosensitivity parameter analysis for the modelled *in vivo* survival obtained using heterogeneous or uniform radionuclide distribution. The sensitivity parameters are either constant or Gaussian distributed. For each α value, the upper and lower dashed lines indicate the upper and

lower 1 SD limit corresponding to α/β=100 Gy and α/β=5 Gy, respectively. The continuous lines correspond to α/β=10.

Δt (days)	Tumor volume nomenclature	Volume (mm^3) (tumor cells %)	S-value (Gy/decay)	Average absorbed dose (Gy) (range)	Heterogeneity*	Average dose in each Δt (Gy)	Dose for homogenous exposure (range) (Gy)
0-2	A1	23.75 (99.70%)	8.78E-10 ± 3.38E-12	3.37 ± 0.01 (0.16 - 7.25)	54.45%	3.57 ± 0.34	3.43± 0.09 (0.00 – 4.92)
	A2	17.44 (96.16%)	1.07E-09 ± 3.40E-12	4.09 ± 0.01 (0.10 - 10.32)	53.19%		
	A3	23.57 (99.40%)	8.82E-10 ± 3.37E-12	3.38 ± 0.01 (0.16 - 8.07)	48.34%		
	A4	22.26 (98.51%)	9.03E-10 ± 3.36E-12	3.46 ± 0.01 (0.03 - 8.70)	50.07%		
2-5	B1	22.39 (98.50%)	9.04E-10 ± 3.39E-12	1.74 ± 0.01 (0.07 – 4.68)	49.02%	1.80 ± 0.18	1.72± 0.05 (0.00 – 2.47)
	B2	15.45 (94.18%)	1.08E-09 ± 3.42E-12	2.08 ± 0.01 (0.03 – 6.94)	45.14%		
	B3	23.27 (98.86%)	8.78E-10 ± 3.38E-12	1.69 ± 0.01 (0.13 – 3.67)	51.94%		
	B4	22.80 (99.06%)	8.95E-10 ± 3.39E-12	1.72 ± 0.01 (0.05 – 4.11)	49.61%		
5-11	C1	22.71 (98.08%)	8.97E-10 ± 3.38E-12	1.57 ± 0.01 (0.07 – 4.34)	40.73%	1.81 ± 0.29	1.57±0.04 (0.00 – 2.25)
	C2	16.39 (99.17%)	1.23E-09 ± 3.43E-12	2.16 ± 0.01 (0.05 – 5.48)	48.51%		
	C3	23.09 (99.31%)	9.01E-10 ± 3.38E-12	1.58 ± 0.01 (0.05 – 4.30)	50.23%		
	C4	19.13 (98.97%)	1.10E-09 ± 3.36E-12	1.92 ± 0.01 (0 – 4.46)	52.52%		
11-14	D1	22.73 (98.76%)	9.07E-10 ± 3.37E-12	1.78 ± 0.01 (0.02 – 4.41)	51.78%	2.23 ± 0.56	1.75±0.05 (0.00 -2.52)
	D2	12.51 (93.69%)	1.35E-09 ± 3.42E-12	2.65 ± 0.01 (0.01 – 9.53)	45.91%		
	D3	12.99 (96.28%)	1.42E-09 ± 3.43E-12	2.77 ± 0.01 (0.12 – 7.69)	43.67%		
	D4	23.93 (99.67%)	8.75E-10 ± 3.39E-12	1.71 ± 0.01 (0.11 – 3.59)	51.66%		
Average			1.01E-09 ± 3.39E-12		49.17% ± 3.72%		
Cumulative (0-14 days)						8.94 ± 2.02	8.46±0.00

** Indicates the percentage of volume exposed to a dose equal or higher than the average value in each tissue section (i.e. previous column).*

SUPPLEMENTAL TABLE 1. Average physical parameters summary for each dissected tissue section. The error is reported as +/- 1 SD.

days 0-2					
a	A1	A2	A3	A4	Sphere
1	3.37	4.09	3.38	3.46	3.39
-1	3.13	3.53	3.06	3.05	0.05
-2	2.96	3.15	2.86	2.73	1.14E-04
-3	2.75	2.73	2.62	2.21	8.82E-06
-4	2.48	2.27	2.33	1.37	2.21E-06
-5	2.16	1.83	2.00	0.77	9.40E-07
-6	1.83	1.43	1.68	0.49	5.28E-07
-7	1.51	1.09	1.38	0.34	3.49E-07
-8	1.25	0.85	1.14	0.26	2.56E-07
-9	1.05	0.69	0.96	0.20	2.00E-07
-10	0.89	0.58	0.82	0.17	1.65E-07
days 2-5					
a	B1	B2	B3	B4	Sphere
1	1.74	2.08	1.69	1.72	1.70
-1	1.51	1.63	1.57	1.52	0.02
-2	1.36	1.34	1.50	1.39	5.73E-05
-3	1.20	1.05	1.40	1.23	4.41E-06
-4	1.02	0.78	1.28	1.03	1.11E-06
-5	0.84	0.56	1.15	0.81	4.70E-07
-6	0.68	0.40	1.01	0.62	2.64E-07
-7	0.55	0.29	0.88	0.47	1.75E-07
-8	0.45	0.23	0.77	0.37	1.28E-07
-9	0.38	0.18	0.67	0.30	1.00E-07
-10	0.33	0.15	0.60	0.25	8.27E-08
days 5-11					
a	C1	C2	C3	C4	Sphere
1	1.57	2.16	1.58	1.92	1.55
-1	1.37	1.98	1.41	1.69	0.02
-2	1.25	1.87	1.30	1.08	5.24E-05
-3	1.11	1.72	1.16	0.15	4.04E-06
-4	0.96	1.50	1.02	0.04	1.01E-06
-5	0.80	1.14	0.88	0.02	4.30E-07
-6	0.67	0.79	0.73	0.01	2.42E-07
-7	0.55	0.56	0.60	0.01	1.60E-07
-8	0.46	0.42	0.47	0.01	1.17E-07
-9	0.40	0.34	0.38	0.00	9.18E-08
-10	0.34	0.28	0.32	0.00	7.56E-08
days 11-14					
a	D1	D2	D3	D4	Sphere
1	1.78	2.65	2.77	1.71	1.73

-1	1.61	2.26	2.37	1.60	0.02
-2	1.49	1.95	2.16	1.52	5.85E-05
-3	1.35	0.98	1.94	1.42	4.51E-06
-4	1.13	0.36	1.71	1.30	1.13E-06
-5	0.77	0.18	1.47	1.15	4.80E-07
-6	0.48	0.11	1.22	0.99	2.70E-07
-7	0.32	0.08	1.00	0.84	1.78E-07
-8	0.24	0.06	0.83	0.72	1.31E-07
-9	0.18	0.05	0.70	0.61	1.02E-07
-10	0.15	0.04	0.60	0.53	8.44E-08

SUPPLEMENTAL TABLE 2. Generalized equivalent uniform dose (gEUD) for the dose distributions reported in Supplemental Figure 5.