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Modelling oxygen effects on the in- and out-of-field radiosensitivity of cells exposed to intensity-modulated radiation fields

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ABSTRACT

Objective: The delivery of intensity-modulated radiation fields has improved the conformity of dose to tumour targets during radiotherapy (RT). Previously, it has been shown that intercellular communication between cells positioned in- and outside of the radiation field impacts cellular radiosensitivity under hypoxic and normoxic conditions. However, the mechanism of intercellular communication in hypoxia remains to be fully understood. In this study, the cell-killing effects of intercellular communication in hypoxia were modelled in an effort to better understand the underlying mechanisms of response.

Approach: By irradiating a 50% area of the culture dish (half-field exposure), experimental dose-response curves for cell survival and residual DNA double-strand breaks (DSBs) were generated in prostate (DU145) and non-small cell lung cancer (H1299) cells. The oxygen enhancement ratio (OER) was determined from early DSB yields (corresponding to relative direct damage) and used to model the in- and out-of-field radiosensitivity.

Main results: The developed *integrated microdosimetric-kinetic (IMK) model* successfully predicted the experimental dose responses for survival and lethal lesions, and provides a mechanistic interpretation that the probability of hits for releasing cell-killing signals is dependent on oxygen. This experimental and modelling study also suggests that residual DSBs correspond to logarithmic survival fraction (meaning lethal lesions) for in- and out-of-field cells. Our data suggest that the OER value determined using uniform-field exposure can be applied to predict the in- and out-of-field radiosensitivity of cells following exposure to intensity modulated beams.

Significance: The developed IMK model facilitates a more precise understanding of intercellular signalling following exposure to intensity-modulated radiation fields.

Keywords: Hypoxia, biophysical model, cell survival, lethal damage, intercellular signaling.

1. Introduction

The efficacy of radiotherapy (RT) is dependent on biological factors that impact the process of cellular repair, redistribution, repopulation and reoxygenation (Wither 1975). Hypoxia

40 is a key factor that drives radioresistance (Garty *et al* 1953) and various strategies have been
41 explored to selectively target hypoxic cells including oxygen-nicotinamide, bioreductive drugs
42 (Laurence *et al* 1995, McKeown *et al* 2007) and fractionated treatment regimens (Sugano *et al*
43 2015) using intensity-modulated RT (IMRT) and volumetric modulated arc therapy (VMAT) that
44 are the established clinical standard in advanced conformal RT (Kuperman *et al* 2008, McGary *et al*
45 2011). During the delivery of modulated RT techniques, the 3-dimensional dose distribution
46 within target tumour volumes is highly heterogeneous at the cellular level and can induce
47 intercellular signalling between irradiated and non-irradiated cells (Prise *et al* 2009, Butterworth
48 *et al* 2011). This intercellular signalling can modulate the radiosensitivity of cells in- and out-of-
49 field through protective (in-field) (Matsuya *et al* 2019 2022) and bystander effects (out-of-field)
50 (Butterworth *et al* 2011, Trainor *et al* 2012, Ghita *et al* 2015). An improved understanding of the
51 mechanisms of cellular response to intensity-modulated radiation fields could potentially lead to
52 the further optimisation of treatments by maximising the probability of tumour control (TCP) and
53 reducing the normal tissue complication probability (NTCP) (Bentzen *et al* 2009).

54 To investigate cellular radiosensitivity under intensity-modulated radiation fields, the
55 oxygen enhancement ratio (OER), defined as the dose ratio between exposure in hypoxia and in
56 air for the same biological endpoint (Hall *et al* 2010) has been evaluated *in vitro*. We have
57 previously shown the impact of hypoxia on out-of-field cell survival after 4 or 8 Gy irradiation
58 appeared to be independent of oxygen concentration (Thompson *et al* 2017). Further experimental
59 studies have shown that hypoxia can have significant effects on out-of-field radiosensitivity that
60 are dependent on the in-field dose. Also, the maximum level of cell killing for out-of-field cancer
61 cells (i.e., DU145 and H1299) after irradiation with high in-field dose is known to be less
62 dependent on oxygen (Matsuya *et al* 2021). Together, these data show that intercellular signalling
63 in hypoxia can enhance out-of-field cell killing, however, responses under hypoxia remain to be
64 fully characterised.

65 Radiobiological studies combined with modelling approaches are an effective approach
66 towards better understanding the potential mechanisms of intercellular signalling (McMahon *et al*
67 2012, Sato *et al* 2018b, Scholz *et al* 2020, Matsuya *et al* 2018 2020a, Monini *et al* 2019).
68 Amongst the various mechanistic models for predicting cell killing, the “integrated
69 microdosimetric-kinetic model (IMK) model” explicitly considers DNA damage kinetics
70 (Matsuya *et al* 2018), intercellular signalling (Matsuya *et al* 2019) and oxygen effects (Matsuya
71 *et al* 2020a). Taking account of these features, further development of the *IMK model* is expected
72 to enable us to mechanistically interpret the scenario of intercellular signalling in hypoxia.

73 In this study, we investigated intercellular signalling in hypoxia from the standpoint of
74 IMK model development. Using a shielding technique where only 50% of the area of a cell culture
75 dish is exposed (i.e., half-field exposure) (Trainor *et al* 2012), we generated experimental dose-
76 response curves for cell survival and residual nuclear DNA damage for prostate cancer cells
77 (DU145) and small cell lung cancer (H1299). Using a common OER value, we modelled the in-
78 field and out-of-field radiosensitivities based on the oxygen-dependent hit probabilities of target

79 DNA and signal release elements. Throughout this study, we propose the dominant impact of
80 intercellular signalling and a theoretical model useful for future predictions in radiation therapy.

81
82

83 **2. Overview of Model Development**

84 *2-1. Assumptions of Oxygen Effects in the IMK model*

85 The biological effects after half-field exposure are believed to be induced by DNA-
86 targeted effects (Hall *et al* 2006) and intercellular signaling (Prise *et al* 2009). A schematic
87 illustration of a half-field exposure setup is shown in Fig. 1(A), and the biological effects from
88 the half-field exposure is illustrated in Fig 1(B). For the targeted effects, in the presence of O₂, the
89 interaction between radiation and liquid water produces several types of free radicals reactive to
90 DNA, e.g., the hydroxyl radical ($\cdot\text{OH}$), leading to DNA-damage (Wardman *et al* 2008, Cadet *et al*
91 2017). Based on this evidence, as shown in the bottom right of Fig.1(B), in our previous modelling
92 of the oxygen effects for DNA-targeted effects, the OER for early DNA double-strand breaks
93 (DSBs) yield was incorporated as the ratio of the yield of potentially lethal lesions (PLLs) under
94 oxygen rich condition ($p_{\text{O}_2} \geq 20\%$) to that under any oxygen pressure, p_{O_2} (%). The yield of PLL
95 k (Gy^{-1}) is defined in the IMK model (Matsuya *et al* 2019). Calculating the ratio of k values under
96 oxygen rich condition and any oxygen pressure, the OER can be expressed by

$$\text{OER}(p_{\text{O}_2}) = \frac{k(\geq 20\%)}{k(p_{\text{O}_2})}, \quad [\because \text{OER}(p_{\text{O}_2}) \geq 1.0] \quad (1)$$

97 where $k(p_{\text{O}_2})$ is the PLL yield under any oxygen pressure and $k(\geq 20\%)$ is the yield under oxygen
98 rich condition. In this model, the oxygen rich condition (normoxia) was set as a fixed point
99 because *in vitro* experiments in air can be easily performed. The coefficients for dose (D) and
100 dose square (D^2) (i.e., α_0 and β_0) in the IMK model for DNA-targeted effects are proportional to
101 OER and OER squared, respectively (Matsuya *et al* 2020a).

102 For intercellular signalling, we previously modelled the probabilities of a given cell having
103 an activated target for emitting cell-killing signals and that of a cell having no activated targets
104 based on microdosimetry, and expressed as the mean number of lethal lesions (LLs) per cell. To
105 consider the oxygen dependence, based on the previous experimental data (Thompson *et al* 2017,
106 Matsuya *et al* 2021), we now make the following assumptions:

- 107 (i) The mean number of targets activated for releasing intercellular signals per hit cell
108 depends on oxygen pressure. The coefficients for D and D^2 for intercellular signals, α_b
109 and β_b , are proportional to OER and OER squared, respectively, as shown in the bottom
110 left of Fig. 1(B).
- 111 (ii) The OER value for DNA-targeted effects (i.e., ratio of early DSB yields) is applied to the
112 model for intercellular signalling, which means that the probability of target activation
113 for releasing cell-killing signals decreases in hypoxia.
- 114 (iii) The parameter representing the LL yield in non-hit cells δ (Matsuya *et al* 2018) is

115 independent of oxygen pressure, which indicates that the maximum number of LLs
 116 induced by intercellular signals per cell is constant.

117 Based on these assumptions for intercellular signalling, we modelled the in-field and out-
 118 of-field radiosensitivities in the same manner as the previous modelling.

119

120 2-2. Surviving fraction of in-field and out-of-field cells

121 Using the above assumptions, we modelled the surviving fractions of in-field and out-of-
 122 field cells based on the IMK model, which was previously developed for half-field exposures
 123 (Matsuya *et al* 2019). The present IMK model is composed of two parts, DNA-targeted effects
 124 and intercellular signalling (so called non-targeted effects).

125 We used the IMK model for DNA-targeted effects considering microdosimetry, sub-lethal
 126 damage repair (SLDR) during irradiation and oxygen effects, which has already been verified
 127 compared to the experimental data (Matsuya *et al* 2018 2019 2020a). The cell surviving fraction
 128 for DNA-targeted effects S_T can be given by

$$\begin{aligned} -\ln S_T = w_T &= (\alpha_0^* + \gamma_* \beta_0^*) \dot{D}T + \frac{2\beta_0^*}{(a+c)^2 T^2} [(a+c)T + e^{-(a+c)T} - 1] (\dot{D}T)^2 \\ &= (\alpha_0^* + \gamma_* \beta_0^*) D + F\beta_0^* D^2 \end{aligned} \quad (2)$$

129 where w_T is the number of LLs (residual lesions) per cell for DNA-targeted effects, \dot{D} is constant
 130 dose-rate in Gy/h; T is dose-delivery time in hour; $(a+c)$ is the sum of the constant rate for a PLL
 131 to transform into a LL and that for DNA repair; γ_* is the microdosimetric quantity ($= y_D/\rho\pi r_d^2$)
 132 (the symbol * stands for either in-field (IF) or out-of-field (OF)); y_D is the dose-mean lineal energy
 133 in keV/ μm (ICRU 1983), r_d and ρ are the radius and density of the microdosimetric site (so called
 134 domain), respectively ($r_d = 0.5 \mu\text{m}$, $\rho = 1.0 \text{ g/cm}^3$) (Hawkins 1996). It should be noted that F
 135 describes the dose-rate effects induced by cell recovery during irradiation, which corresponds to
 136 the Lea-Catcheside time factor (Brenner 2008). The cell-specific parameters α_0^* and β_0^* are the
 137 coefficients to D (Gy^{-1}) and D^2 (Gy^{-2}). These coefficients depend on radiation field type even in
 138 case of the same cell line because of protective effects (intercellular communication). In our
 139 previous modelling, the protective effects were simply considered using the ratio of PLL yield
 140 under certain field type and uniform-field exposures, $\varphi_{PE} = k_{\text{some}}/k_{\text{uniform}}$ (Matsuya *et al* 2019),
 141 where k_{some} is the PLL yield for certain field-type exposure and k_{uniform} is the yield for uniform-
 142 field exposure. Considering the oxygen effects $\text{OER}(p_{\text{O}_2})$ and the yield modification by radiation
 143 field type φ_{PE} , α_0^* and β_0^* are expressed by

$$\alpha_0^* = \frac{\alpha_0 \varphi_{PE}}{\text{OER}(p_{\text{O}_2})} \quad \text{and} \quad \beta_0^* = \frac{\beta_0 \varphi_{PE}^2}{\text{OER}(p_{\text{O}_2})^2} \quad (3)$$

144 where α_0 and β_0 were the coefficients for uniform-field exposure in normoxia. These coefficients
 145 were newly defined based on previous models of oxygen effects and the protective effects induced
 146 under modulated field exposure. In addition, the correction factor to consider the protective effects,

147 φ_{PE} , is newly defined in this modelling. This consideration of the OER value into the coefficients
 148 to dose and dose square can be linked to the previous modelling for oxygen effects based on the
 149 Linear-Quadratic (LQ) model (Carlson *et al* 2006).

150 The surviving fraction for intercellular signalling is modelled based on the previous
 151 modelling, which has been also verified compared to the experimental data (Matsuya *et al* 2019).
 152 In the same manner as DNA-targeted effects, $OER(p_{O_2})$ was incorporated into the model for
 153 intercellular signalling. The surviving fraction for intercellular signalling is expressed by

$$-\ln S_{IS} = w_{IS} = \delta \left[1 - e^{-(\alpha_b^* + \gamma_{IF}\beta_b^*)D_{IF} - \beta_b^* D_{IF}^2} \right] e^{-(\alpha_b^* + \gamma_{*}\beta_b^*)D^* - \beta_b^* D^{*2}} \quad (4)$$

154 where S_{IS} is the surviving fraction for intercellular signalling (IS); α_b^* and β_b^* are cell-specific
 155 coefficients to D^* and D^{*2} , respectively (* stands for either in-field (IF) or out-of-field (OF)); δ is
 156 the yield of lethal lesions (LLs) in non-hit cells. In the same manner as DNA-targeted effects, the
 157 coefficients (α_b^* and β_b^*) includes $OER(p_{O_2})$ defined in Eq. (1), which are expressed by

$$\alpha_b^* = \frac{\alpha_b}{OER(p_{O_2})} \quad \text{and} \quad \beta_b^* = \frac{\beta_b}{OER(p_{O_2})^2}. \quad (5)$$

158 Assuming that the interaction probability between sub-lesions (PLLs) induced by DNA
 159 targeted effects and intercellular signalling is very small (Sato *et al* 2014, Matsuya *et al* 2018),
 160 overall surviving fraction S can be expressed by

$$S = S_T \times S_{IS}. \quad (6)$$

161 The cell-specific parameters ($\alpha_0, \beta_0, (a+c), \alpha_b, \beta_b, \delta, \varphi_{PE}$) can be obtained from applying the model
 162 to the dose-response curve of cell survival in normoxia. γ can be determined from Monte Carlo
 163 simulation for radiation transport. OER can be obtained from ratio of DSB yields in normoxia and
 164 hypoxia or applying the model to the experimental survival data. Using Eqs. (1-6), we investigated
 165 the scenario of intercellular signalling in hypoxia.

166

167 **3. Materials and Methods**

168 *3-1. Cell culture*

169 To verify the developed model, we used two cancer cell lines, human prostate cancer
 170 (DU145) (RIKEN Science Institute BRC: Ibaraki, Japan), and non-small cell lung cancer (H1299)
 171 (ATCC: Manassas, VA, USA). DU145 cells were cultured in RPMI-1640 medium (Thermo Fisher
 172 Scientific Inc. Tokyo, Japan) with 10% fetal bovine serum (FBS, Nichirei Bioscience Inc., Tokyo,
 173 Japan) and 1% penicillin/streptomycin (p/s). H1299 cells were cultured in Dulbecco's Modified
 174 Eagle Medium (DMEM, Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10% FBS
 175 and 1% p/s. Both DU145 and H1299 cells were maintained at 37 °C in a humidified atmosphere
 176 of 95% air/5% CO₂.

177

178 *3-2. Hypoxic treatment*

179 A nBIONIX-2 hypoxic cell culture kit (Sugiyamagen: Tokyo, Japan) (Kaida *et al* 2012)
180 was used to induce hypoxic conditions *in vitro*. A cell culture dish containing the cultured cells
181 and an AnaeroPack (oxygen absorber; Mitsubishi Gas Chemical, Tokyo, Japan) were placed inside
182 a gas barrier pouch bag (Mitsubishi GasChemical) 4 h prior to irradiation. After placement, the
183 oxygen concentration inside the pouch bag was continuously monitored until the sensitivity
184 threshold of the OXY-2 oxygen monitor (JIKCO, Tokyo, Japan), which was 0.0% O₂. The
185 radiobiological level of hypoxia was < 0.4% O₂ (McKeown *et al* 2014). After this hypoxic
186 treatment, the cells were irradiated. After irradiation, the flasks were returned to normoxia.

187

188 3-3. Irradiation

189 Cells were irradiated with 150 kVp X-rays (1 mm Al filtration and 1.82 Gy/min)
190 generated from an X-ray generator (MBR-1520R, Hitachi Medical Co., Tokyo, Japan). By
191 shielding 50% area of a cell culture container, the dose was delivered to either 50% of the area of
192 a culture container (so called half-field exposure) or 100% of the container (so called uniform-
193 field exposure). As shown in the bottom of Fig. 1A, the dose profile in the half-field exposure was
194 evaluated by the Monte Carlo simulation and the measurement with Gafchromic film as reported
195 previously (Matsuya *et al* 2021), in which the out-of-field dose 1.0 cm away from dose boundary
196 between in-field area and out-of-field one is 2.3% of the in-field doses.

197

198 3-4. Detection of residual DSB sites

199 The irradiated cells were fixed in 4% paraformaldehyde for 10 min on ice 24 h after
200 irradiation. The fixed cells were permeabilized using 0.2% v/v Triton X-100 in phosphate buffered
201 saline (PBS) for 5 min. The cells were then blocked in 1% bovine serum albumin (BSA) in PBS
202 for 1 h. The cells were then incubated at 4°C overnight with primary antibodies against γ -H2AX
203 (ab26350, Abcam) diluted 1:400 by the 1% BSA in PBS. After rinsing with 1% BSA in PBS three
204 times, the cells were incubated in the dark at room temperature for 2 h with secondary antibodies
205 Alexa Fluor 594-conjugated goat-anti-mouse IgG H&L (ab150116, Abcam) diluted 1:250 by a
206 1% BSA in PBS. After rinsing with the 1% BSA in PBS three times, the cells were incubated in
207 the dark with 1 μ g/ml DAPI solution (62248, Thermo Fisher Scientific) for 15 min. After rinsing
208 once with methanol, we observed γ -H2AX foci using a Keyence BZ-9000 fluorescent microscope
209 (Keyence, Osaka, Japan). The nuclear foci were evaluated with the automated foci counting
210 module for peak detection using ImageJ software (Rasband *et al* 1997–2007, Abramoff *et al* 2004).
211 The radiation-induced number of foci was further calculated by the subtraction of the number of
212 background foci in non-exposed cells. The experiments were repeated four times and the standard
213 error of the mean (s.e.m) was obtained.

214

215 3-5. Analysis of cell survival data

216 Using the experimental cell survival data for acute irradiation reported previously
217 (Thompson *et al* 2017, Matsuya *et al* 2019 2021), we obtained a set of model parameters (α_0 , β_0 ,

218 $(a+c)$, γ , α_b , β_b , δ , φ_{PE}) for several cell models including DU145 prostate cancer cells, and H1299
219 and H460 lung cells. Note that the experimental survival data of H460 was used to check that the
220 model works irrespective of the cell line type and hypoxic system that generated the experimental
221 data. When determining the parameters, we used two Monte Carlo simulations: one is a track-
222 structure simulation for determining microdosimetric quantity γ and the other is the Markov chain
223 Monte Carlo (MCMC) simulation (Gelman *et al* 2014, Matsuya *et al* 2017) for determining cell-
224 specific parameters $(\alpha_0, \beta_0, (a+c), \alpha_b, \beta_b, \delta)$ including the uncertainties. The procedures are
225 described below.

226 First, the φ_{PE} values for DU145 and H1299 were obtained from the number ratio of the
227 visible γ -H2AX foci 30 min after irradiation (i.e., the number ratio of foci under certain field type
228 and that under uniform field) (Matsuya *et al* 2019). Second, the in-field and out-of-field
229 microdosimetric qualities of γ in Gy was calculated by the radiation track-structure simulation
230 with Particle and Heavy Ion Transport code system (PHITS) version 3.27 for X-rays (Sato *et al*
231 2018a) and WLTrack code for secondary electrons (Date *et al* 2007). The simulation accuracy for
232 microdosimetry calculation was verified in our previous simulation (Matsuya *et al* 2019). Third,
233 the in-field and out-of-field γ values and cell surviving fraction for half-field exposure (due to the
234 largest number of experimental data) were used to determine the parameter set of $(\alpha_0, \beta_0, (a+c)$,
235 $\alpha_b, \beta_b, \delta)$ by the MCMC simulation. In this procedure, the φ_{PE} value for H460 cell line was
236 simultaneously determined from the MCMC simulation due to no experimental γ -H2AX focus
237 data being available. In the MCMC simulation, the uncertainty for $-\ln S$ was assumed to follow a
238 normal distribution. The prior distributions (mean and standard deviation of the IMK model
239 parameter) of DU145 cell line, which is necessary for the MCMC simulation, were obtained from
240 our previous study (Matsuya *et al* 2019), and the parameters were updated by fitting to the cell
241 survival data using MCMC. Similarly, for H1299 and H460, the previously determined
242 distributions of the coefficients to doses for DNA-targeted effects (α_0, β_0) based on the Linear-
243 Quadratic (LQ) model (Matsuya *et al* 2021) were used to efficiently determine the parameter set.
244 The prior distributions of $(a+c)$ for lung cancer cells were assumed to be normally distributed as
245 2.218 ± 0.401 (Matsuya *et al* 2017). Note that the $(a+c)$ value depends on cell-cycle distribution
246 (Matsuya *et al* 2020a). The other parameters were assumed to follow a uniform distribution due
247 to no prior information.

248 Using the determined model parameters, we estimated the dose response of the surviving
249 fraction after acute irradiation based on Eqs. (1-6). The $OER(p_{O_2})$ values defined in Eq. (1) were
250 obtained from the OER for the DSB detected at 30 min after irradiation of 1 Gy (Matsuya *et al*
251 2021). As no experimental DSB data was available for the H460 cell line, the OER value for the
252 survival endpoint (Thompson *et al* 2017) was used for the model prediction. The number of LLs
253 (residual lethal lesions) per cell was also estimated by the IMK model (Eqs. (1-6)). The estimated
254 dose responses of surviving fraction and nuclear LLs were compared to the corresponding
255 experimental data measured in this study and the previous study (Thompson *et al* 2017, Matsuya
256 *et al* 2021).

257

258 3-6. Statistical analysis

259 To evaluate the fit quality of the developed model, we calculated the coefficient of
260 determination R^2 as statistical measures. The R^2 value used in this study is given by

$$R^2 = 1 - \frac{\sum_{i=1}^n (\text{exp}_i - \text{cal}_i)^2}{\sum_{i=1}^n (\text{exp}_i - \langle \text{exp} \rangle)^2}, \quad (7)$$

261 where exp_i is the measured data of surviving fraction or nuclear LLs per cell, cal_i is the estimation
262 by the model, and $\langle \text{exp} \rangle$ is the mean experimental value. Note that log-transformed values were
263 used when evaluating the fit quality for cell survival.

264

265 4. Results and Discussions

266 4-1. Estimation of cell survival in hypoxia

267 The IMK model considering oxygen effects for the half-field exposure was applied to the
268 experimental dose-response curves for the survival of DU145, H1299 and H460 cells in normoxia.
269 The model parameters were determined and are summarized in Table 1. Using the model
270 parameters listed in Table 1, we estimated the dose response for in-field and out-of-field cells.

271 Figure 2 compares the survival curves between the prediction by the IMK model and the
272 experimental data of DU145, H1299 and H460 (Thompson *et al* 2017, Matsuya *et al* 2021), in
273 which (A) is the curves after the uniform-field (UF) cells, (B) is those of in-field (IF) cells after
274 the half-field exposure, and (C) is those of out-of-field (OF) cells after the half-field exposure.
275 The dose-mean linear energy y_D values calculated by the Monte Carlo codes were summarized in
276 Table 2, from which the γ values for in-field and out-of-field are 0.946 and 0.950, respectively.
277 The higher y_D value of out-of-field compared to in-field value is attributed to the scattered X-rays
278 from shielding materials (i.e., Pb). In Fig. 2, we compared the model prediction to the
279 experimental survival of H460 cells to check that the model works irrespective of the experimental
280 conditions (such as cell line type and hypoxic system) that generated the experimental data. As
281 shown by the blue lines in Fig. 2, the dose response curves of in-field and out-of-field cells in
282 normoxia can be reproduced by Eqs. (2), (4) and (6). The out-of-field responses were successfully
283 reproduced by three cell-specific parameters ($\alpha_b, \beta_b, \delta$). Meanwhile, when estimating the surviving
284 fraction of in-field cells uniformly exposed, we used the cell-specific coefficients (α_0, β_0)
285 considering the change of PLL yields between half field and uniform field by the protective effects
286 (Matsuya *et al* 2019), that were $\varphi_{\text{PE}} = 0.936 \pm 0.084$ for DU145 cells, 0.941 ± 0.125 for H1299
287 cells, 0.944 ± 0.236 for H460 cells (see Table 1). Comparisons of the in-field dose response
288 between the different field types (i.e., uniform field and half field) are described in Fig. S1 (see
289 supplementary material) in which the reduced radiosensitivity of in-field cells after the half-field
290 exposure was successfully reproduced by the IMK model.

291 Using the OER value and cell-specific parameters ($\alpha_0, \beta_0, (a+c), \alpha_b, \beta_b, \delta$), the in- and out-

292 of-field cell survival curves in hypoxia were predicted and are shown as the red lines in Fig. 2. To
293 estimate surviving fraction in Fig. 2, we used two OER values. One is 2.31 obtained from the γ -
294 H2AX foci data, and the other is 1.32 calculated from the ratio of doses during hypoxia compared
295 to normoxia leading to 10% survival because the experimental initial DSB yields for H460 was
296 not available. As these hypoxic conditions were created by either nBIONIX-2 or gas-exchanging
297 system, the OER value is different each other. For the irradiation condition using the gas-exchange
298 approach, we irradiated the DU145 cells in air after treatment in the nBIONIX-2 system. When
299 the cells were exposed to oxygen during irradiation, the impact of hypoxia on radiosensitivity was
300 reduced (see Fig. S2 in supplementary materials). These findings are similar to those in a previous
301 report (Thompson *et al* 2017). However, both deliver sufficient hypoxia from radiobiological
302 standpoint (McKeown *et al* 2014). The agreement between the IMK model predictions and the
303 experimental survival curves under hypoxia (R^2 values for DU145, H1299 and H460 were 0.997,
304 0.994, 0.988, respectively, see Fig. 2) suggests that the model assumption that the hit probability
305 for releasing cell-killing signals (i.e., intercellular signals) depends on oxygen concentration is
306 reasonable. The same OER values were used for the uniform-field and half-field exposure in this
307 study. In this regard, the model analysis suggested that the conventional OER value determined
308 using uniform-field exposures can be applied when predicting both in- and out-of-field
309 radiosensitivity of cells following exposure to intensity modulated beams.

310 To further verify the model for predicting out-of-field cell death, we also compared the
311 model prediction with the experimental survival after at 8 Gy without intercellular signalling (IS).
312 Figure 3 shows the comparison between the prediction by the IMK model with and without IS
313 and the corresponding experimental data of DU145, H1299 and H460 at two conditions with OER
314 = 1.32 and 2.31 (Thompson *et al* 2017, Matsuya *et al* 2021). We assumed that $\delta = 0$ when
315 estimating the surviving fraction without IS, based on our previous model study (Matsuya *et al*
316 2019). From Table 2, the microdosimetric quantities represented as γ for in-field and out-of-field
317 by 225 kVp X-rays were 0.895 and 0.972, respectively. In Fig. 3, the experimental out-of-field
318 survival without intercellular signalling (IS) were measured by administrating 100 μ M
319 aminoguanidine, which is an inhibitor for inducible nitric oxide synthase (iNOS) (Matsuya *et al*
320 2021), or by irradiating with physical inhibition of cell-to-cell communication (in other word,
321 100% out-of-field cells) (Thompson *et al* 2017). As a result, with good agreement between the
322 estimation and the experiment, the clonogenicities of out-of-field cells without IS were higher
323 than those with the IS. iNOS down-regulates nitric oxide (NO). Therefore, the results suggested
324 that cell-to-cell communication is significant and nitric oxide (NO) is dominant species for out-
325 of-field cell killing even under hypoxia.

326 To date, the enhanced radiosensitivity of out-of-field cells (i.e., cell death and DNA lesions)
327 in hypoxia can be attributed to bystander responses (Matsuya *et al* 2022). NO is a dominant factor
328 leading to by cell death of bystander cells (Fig. 3) and can be regulated by the NF- κ B pathway
329 (which relates to inflammation) (Calveley *et al* 2005, Hamada *et al* 2011, Hei *et al* 2011). Taking
330 account of these signalling pathways, inflammatory signalling may play a key role in out-of-field

331 radiosensitivity. Meanwhile, the reduction of radiosensitivity of in-field cells can be interpreted
332 as protective effects by a reduction of early DNA damage (Matsuya *et al* 2019 2022) or rescue
333 effects by stimulated DNA repair (Volcic *et al* 2012, Li *et al* 2019, Pathikonda *et al* 2020). The
334 underlying mechanisms for the protective effects (as well as rescue effects) are still under
335 investigation (Yu *et al* 2022), so further *in vitro* and *in vivo* studies are needed in future. Focusing
336 on the impact of hypoxia, the radiosensitivities of out-of-field cells in hypoxia were found to be
337 reduced compared to normoxia but dependent on the in-field absorbed dose (Thompson *et al* 2017,
338 Matsuya *et al* 2021).

339 We also investigated the relationship between cell survival under normoxia and that under
340 hypoxia using the IMK model, as shown in Fig. S3 (see supplementary material). The relationship
341 estimated by the IMK model showed that hypoxia has only a small impact on out-of-field cells
342 when in-field cells are exposed to a high dose. From these model predictions for cell survival, it
343 is suggested that the role of the intercellular signalling (inflammatory responses) under hypoxia
344 is similar to that in normoxia. From the model analysis, the differences between hypoxia and
345 normoxia is interpreted to be due to the reduced probability for releasing cell-killing signals.

346

347 4-2. Application of the IMK model to residual DSBs

348 Radiation-induced cell killing is related to DSB induction. To further verify the
349 developed model, we applied the model to experimental measurements of residual DSBs (LLs)
350 after irradiation. In this study, we used the γ -H2AX foci formation assay to measure residual DSBs.
351 Using the model parameters listed in Table 1, we then estimated the dose-response curves for in-
352 field and out-of-field LLs per cell, and compared them to the experimentally determined nuclear
353 γ -H2AX foci measured 24 h after irradiation.

354 Figure 4 compares the residual DSBs estimated by the IMK model and the experimental
355 data obtained in this study where (AI) and (AII) are the curves in normoxia and (BI) and (BII) are
356 those in hypoxia. The solid line and symbol are the estimation by the IMK model and the
357 experimental data, respectively. The distribution of nuclear residual foci per cell are shown in Fig.
358 S4 (see supplementary material), where increases for the unrepaired DNA lesions can be observed in
359 normoxia. This experimental distribution shown in Fig. S4 may be valuable for the future
360 theoretical analysis considering cell-cycle distribution (Mori *et al* 2018). From these comparisons,
361 the reductions of the residual DSB induction for both in-field and out-of-field cells were
362 reproduced by the IMK model with good agreement. In particular, the dose responses for in-field
363 cells followed a linear-quadratic response, while those of out-of-field cells exhibit a sigmoidal
364 response as a function of in-field dose, which represents the hit probability for releasing cell-
365 killing signals defined in the model assumption (see Fig. 1B).

366 As shown in Fig. 4, the residual DSBs can be related to the cell-killing induction with a
367 certain probability (Carante *et al* 2015). In this regard, we also depicted the relationship between
368 the residual nuclear DSBs 24 h after irradiation and the corresponding surviving fraction, which
369 was shown in Fig. 5. Figures 5(A) and 5(B) show the relationship for in-field cells and out-of-

370 field cells, respectively. These results suggest that the correlation coefficient for out-of-field cells
371 was lower than that for in-field cells (i.e., R^2 for out-of-field cells and in-field cells are 0.685 and
372 0.953, respectively), which is due to the experimental uncertainty. The model prediction for this
373 relationship in Fig. 5(C) was derived based on the assumption that the number of LLs per nucleus
374 follows a Poisson distribution (i.e., $-\ln S = w = w_T + w_{IS}$), which agreed well with the experimental
375 relationship considering the experimental uncertainties ($R^2 = 0.965$). This tendency is the same as
376 that obtained in previous efforts for interpreting the relationship between residual lesions and
377 surviving fraction (Menegakis *et al* 2009, Olive *et al* 2011). In particular, in this study, it was
378 found that the relationship of $-\ln S = w$ can be applied to, not only the conventional in-field
379 responses, but also the out-of-field responses. In general, the phosphorylation expression of
380 H2AX appears to be higher than the actual unrepaired DSBs, meaning the focus intensity does
381 not correspond one-to-one to DSB (Rothkamm *et al* 2009). One limitation of this study is the use
382 of single marker of DSB γ -H2AX, so the further experiments combined with 53BP1 is needed.
383 Finally, considering the experimental limitations and the overall agreement with the experimental
384 data (Figs. 2–5), the cellular mechanisms (i.e., oxygen-dependent hit probabilities inducing DNA
385 targeted effects and intercellular communication) assumed in the presented model are reasonable,
386 showing a good performance in reproducing biological impacts of in-field and out-of-field cells
387 in hypoxia.

388

389 5. Conclusions

390 In this study, we modelled the cell-killing effects for intercellular communication under
391 hypoxia. By using an oxygen enhancement ratio (OER) defined from DNA-targeted effects of
392 early DSB yields (corresponding relative hit events), the present cell-killing model reproduced
393 the experimental in- and out-of-field radiosensitivities considering intercellular signalling (i.e.,
394 the bystander effects on out-of-field effects and the protective effects on in-field cells). This model
395 provides further interpretation of the role of intercellular communication in hypoxia showing that
396 the yield of lethal DNA lesions in responding cells under hypoxia is lower than that in normoxia,
397 and that the probability of hits for releasing cell-killing signals is dependent on oxygen. The
398 modelling study indicates that the model analysis suggested that the conventional OER value
399 determined using uniform-field exposure can be applied when predicting the in- and out-of-field
400 radiosensitivity of cells following exposure to intensity modulated beams. These findings could
401 contribute to a more precise understanding of intercellular signalling under heterogeneous
402 exposure to intensity-modulated radiation fields.

403 The modelling of the radiobiological effects is a research topic of significant interest. Past
404 models have been developed based on the experimental data *in vitro* with uniform radiation fields.
405 However, from the recent experimental evidence, current estimation approaches based on the
406 model parameters for uniform fields might be insufficient for predicting the responses to advanced
407 radiotherapies using modulated beams. More advanced models for predicting curative effects of
408 cancer-selective treatment such as boron neutron capture therapy (BNCT) and internal

409 radiotherapy with alpha emitters (Sato et al 2018b 2021, Matsuya *et al* 2020b) are also required.
410 Finally, to define the impacts on tumors as well as side effects on normal tissues (Sato *et al* 2022),
411 the accumulation of both experimental data *in vitro* and *in vivo* and modelling approaches is key
412 to future progress.

413

414 **CONFLICT OF INTEREST**

415 The authors declare that they have no conflict of interest.

416

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419 no. 19K17215, 22H03744).

420

421 **SUPPLEMENTARY MATERIALS**

422 The following are available online:

- 423 ▪ Figure S1: Comparison of in-field cell survival between uniform field and half field
- 424 ▪ Figure S2: Radiosensitivity for various hypoxic cell culture systems
- 425 ▪ Figure S3: Relationship between cell survival under normoxia and that under hypoxia
- 426 ▪ Figure S4: Distribution of residual nuclear foci per cell

427

428 **AUTHOR CONTRIBUTIONS**

429 Y. Matsuya, S.J. McMahon and K.M. Prise designed this study. K.T. Butterworth provided the
430 experimental ideas using half-field exposure. Y. Matsuya, Y. Yachi and R. Saga performed the
431 cell experiments. Y. Matsuya and R. Saga developed the present IMK model. Y. Matsuya wrote
432 the manuscript. K.M. Prise and T. Sato supervised this study. All authors reviewed the manuscript.

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577 **Figure captions:**

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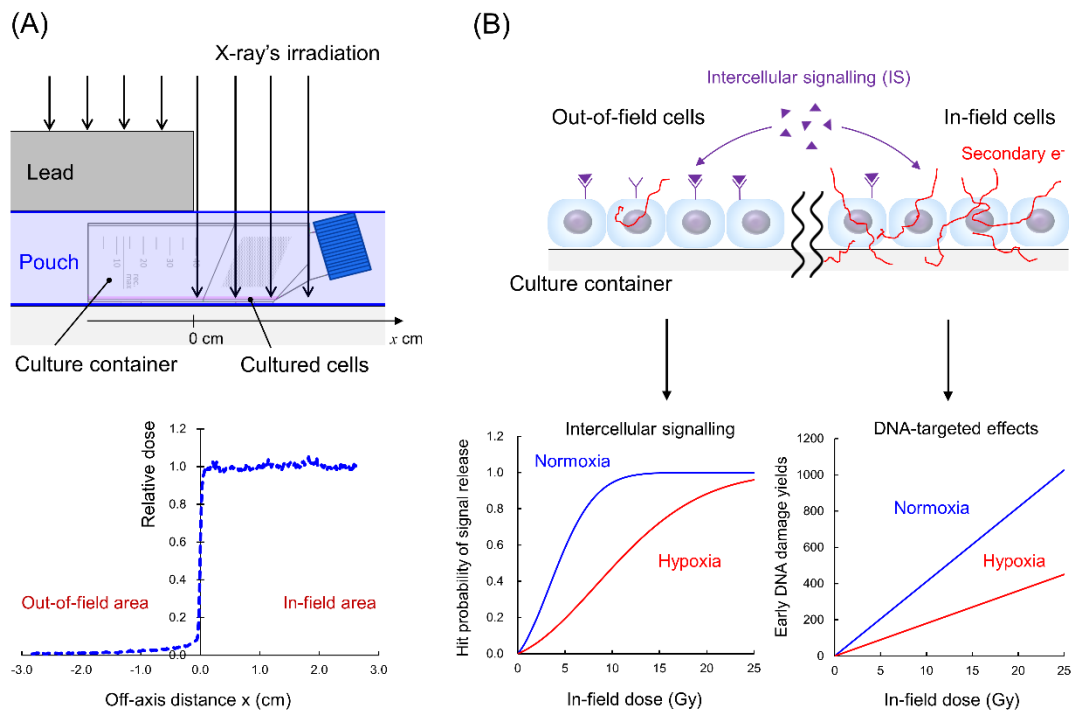
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585 **Figure 1. Schematic illustration of half-field exposure and the biological effects:** (A) is the
586 experimental geometry of the half-field exposure in which by shielding 50% of a cell culture flask
587 was irradiated. The experimental dose profile is given in our previous report (Matsuya *et al* 2021).
588 (B) is the model for oxygen effects for DNA-targeted effects and intercellular signalling. The
589 oxygen dependence on early DNA lesion yields was incorporated into the modelling for the DNA-
590 targeted effects, which has been developed previously (Matsuya *et al* 2020a), while the oxygen-
591 dependent hit and non-hit probabilities for releasing cell-killing signals was newly considered in
592 this IMK model.

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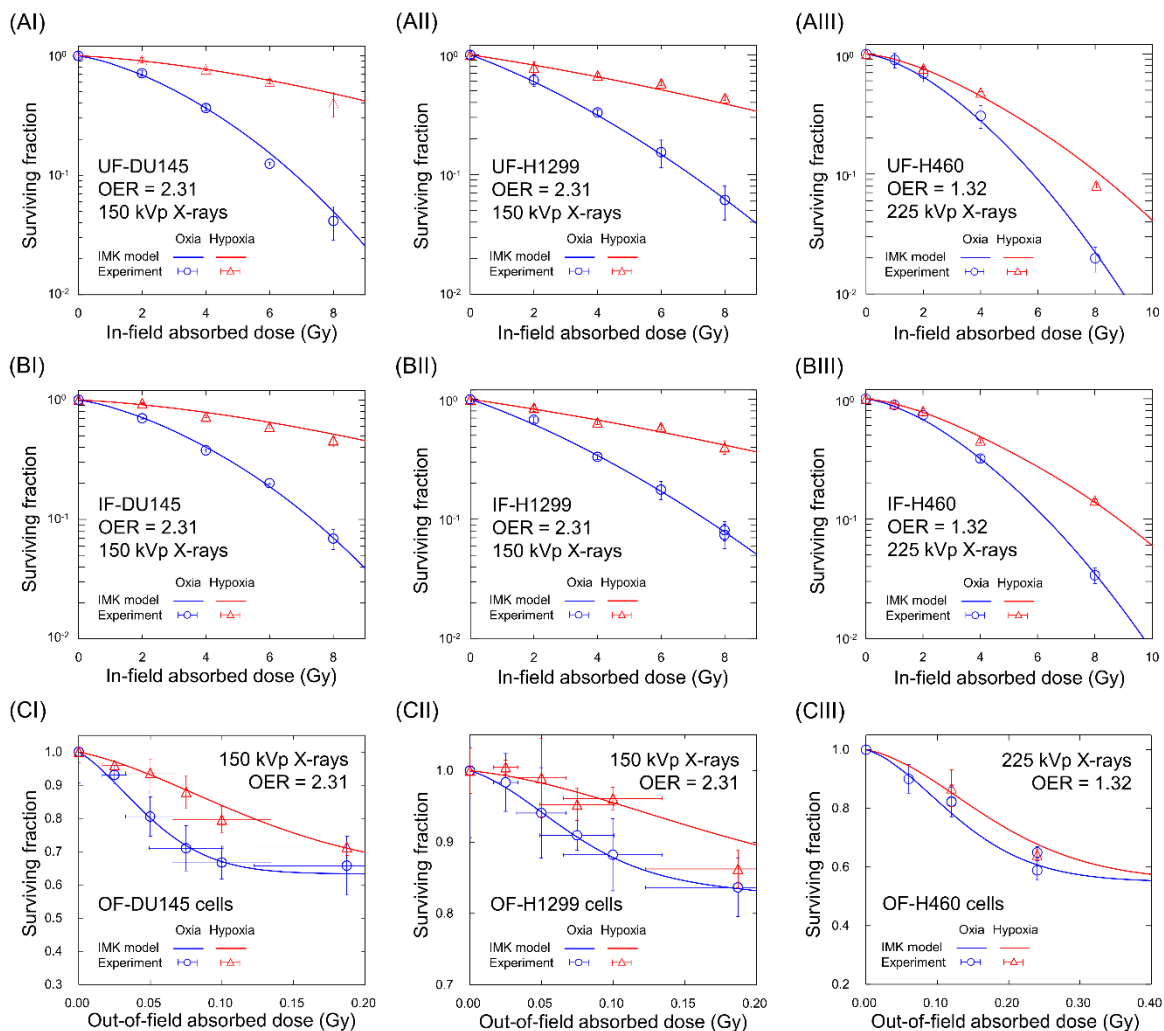
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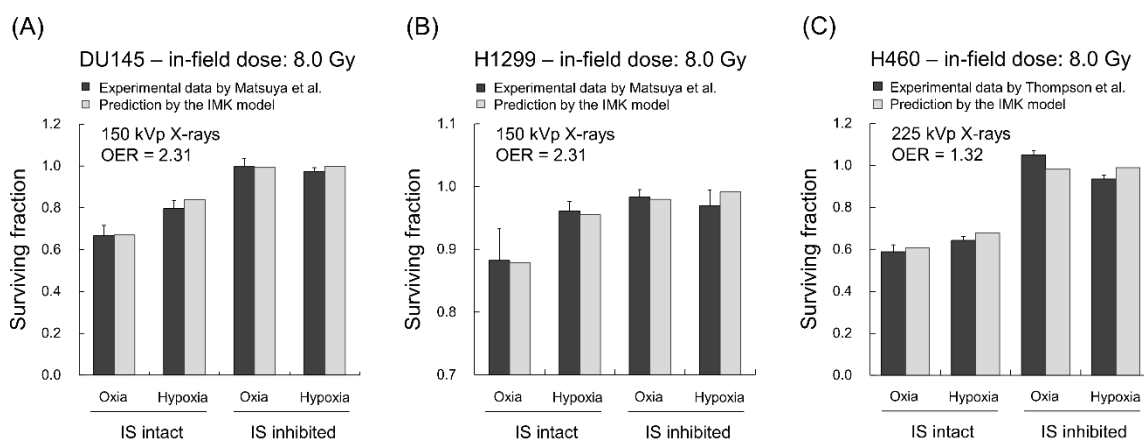


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605 **Figure 2. Dose-response curve of cell survival:** (A) is the curves after uniform-field (UF)
606 exposure, (B) is those of in-field (IF) cells after the half-field exposure, and (C) is those of out-
607 of-field (OF) cells after the half-field exposure. The left panels are the curves of DU145, the
608 central those are H1299 and the right those are H460. The line and the symbol represent the
609 prediction by the IMK model and the experimental data reported in our previous studies
610 (Thompson *et al* 2017, Matsuya *et al* 2021), respectively. Note that the experimental surviving
611 fractions were calculated by the ratio of plating efficiency of irradiated group to that of the non-
612 irradiated group (control cells). The out-of-field dose for 225 kVp and 150 kVp X-rays are 3.00%
613 and 1.25% of in-field dose, respectively. The in-field dose rates for 150 kVp X-rays and 225 kVp
614 X-rays were 1.82 and 0.591 Gy/min, respectively. The R^2 values for DU145, H1299 and H460
615 were 0.997, 0.994, 0.988, respectively.

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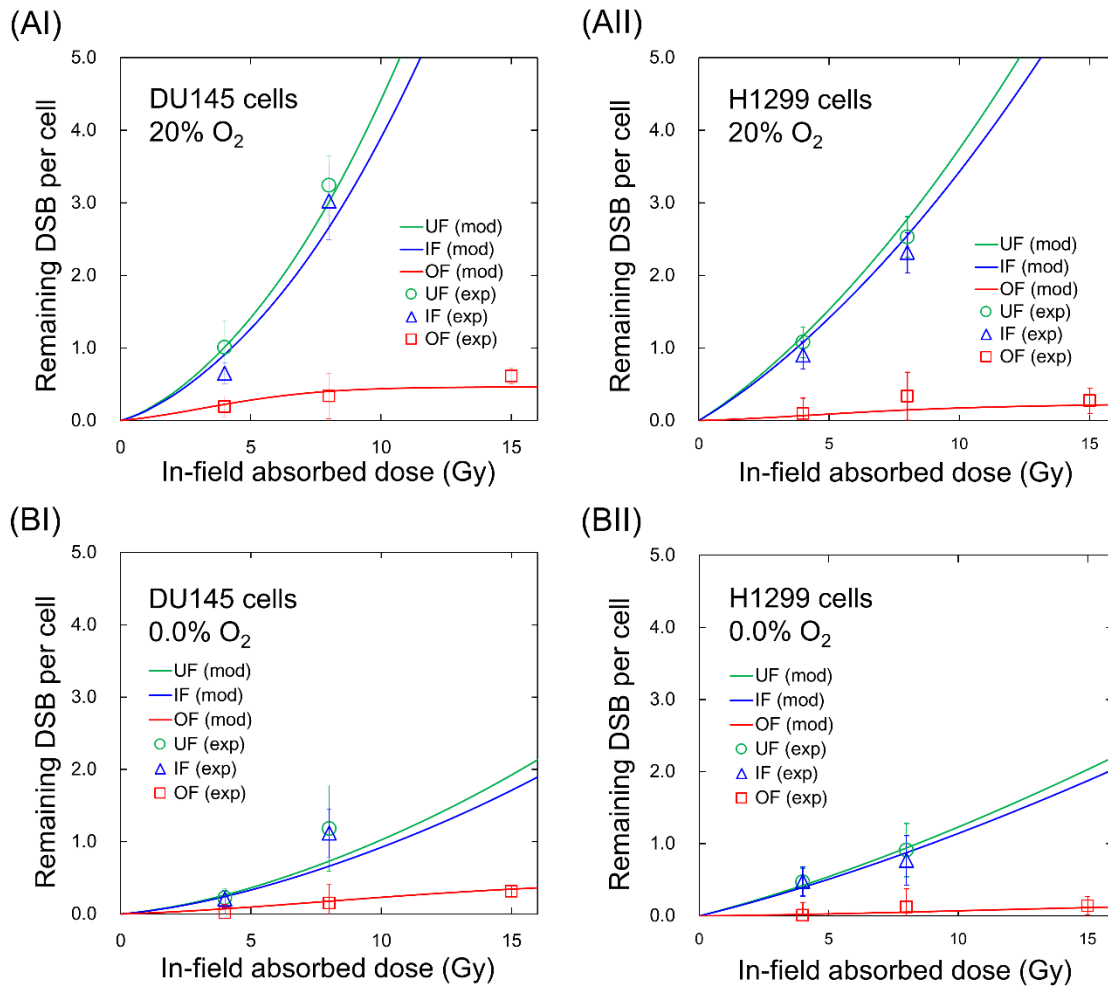
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Figure 3. Out-of-field surviving fraction after 8 Gy irradiation with and without intercellular signals: (A) is the DU145 for OER = 2.31, (B) is the H1299 for OER = 2.31, (C) is the H460 for OER = 1.32. The IMK model agreed well with the experimental data (Thompson *et al* 2017, Matsuya *et al* 2021). We assumed that $\delta = 0$ when estimating the surviving fraction without IS, based on our previous model study (Matsuya *et al* 2019). The out-of-field dose for 225-kVp and 150-kVp X-rays are 3.00% and 1.25% of in-field dose, respectively. The in-field dose rates were 1.82 Gy/min for 150 kVp and 0.591 Gy/min for 225 kVp.

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653 **Figure 4. Residual DSBs 24 after irradiation:** (AI) and (AII) are the curves in normoxia, and
654 (BI) and (BII) are those in hypoxia. The left panels are the curves of prostate cancer cells DU145
655 and the right those are non-small cell lung cancer H1299. Solid line and symbol are the estimation
656 by the IMK model (based on Eqs (1-6)) and the experimental data measured by the γ -H2AX focus
657 formation assay. The out-of-field dose is 2.28% of in-field dose. In the same manner as Fig. 2, the
658 in-field dose rate was 1.82 Gy/min. The R^2 values for DU145 and H1299 were 0.964 and 0.970,
659 respectively.

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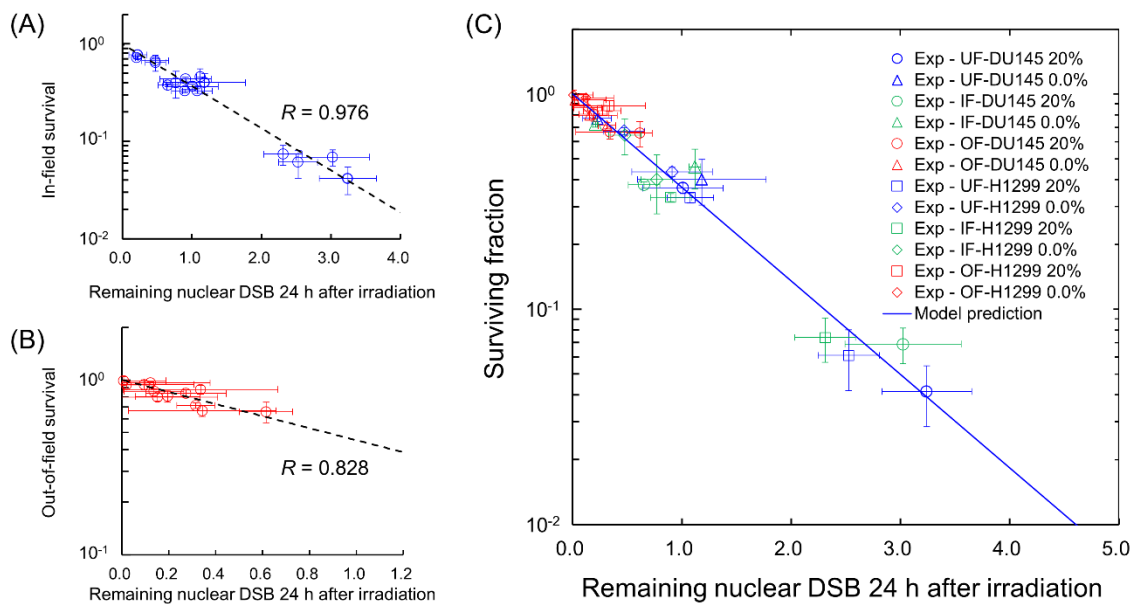
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677 **Figure 5. Relationship between the remaining nuclear DSB 24 h after irradiation and**
 678 **surviving fraction:** Solid line and symbol are the estimation by the IMK model ($-\ln S = w = w_T$
 679 $+ w_{IS}$) and the experimental data measured by the γ -H2AX focus formation assay. OF, IF and UF
 680 mean out-of-field for half-field exposure, in-field for half-field exposure and uniform-field
 681 exposure, respectively. This comparison between model prediction and the experimental data
 682 proves that the residual DSB can be linked to the cell-killing induction with a certain probability.

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Table 1. Cell-specific model parameters in the IMK model for oxic condition

Effect type	Parameters	Cell line type			Unit
		DU145	H1299	H460	
DNA-targeted effects	α_0	0.035 ± 0.007	0.203 ± 0.027	0.020 ± 0.015	Gy^{-1}
	β_0	0.039 ± 0.005	0.017 ± 0.003	0.058 ± 0.007	Gy^{-2}
	$a+c$	2.092 ± 1.306	2.207 ± 0.401	2.222 ± 0.389	h^{-1}
Protective effects	$\varphi_{\text{PE}}^{\text{a,b}}$	0.936 ± 0.084	0.941 ± 0.125	0.944 ± 0.236	-
Intercellular signalling	α_b	0.045 ± 0.024	0.017 ± 0.018	0.011 ± 0.037	Gy^{-1}
	β_b	0.025 ± 0.006	0.018 ± 0.007	0.025 ± 0.010	Gy^{-2}
	δ	0.450 ± 0.048	0.144 ± 0.025	0.571 ± 0.086	-

^a These values for DU145 and H1299 were obtained from yield ratio of γ -H2AX foci (Matsuya *et al* 2021), while that for H460 was determined by the MCMC simulation.

^b The φ_{PE} values presented in this table for half-field exposure. When obtaining α_0^* and β_0^* for the uniform field exposure, we set φ_{PE} to be 1.00.

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Table 2. Microdosimetric quantities calculated by Monte Carlo simulations

Radiation type	Shielding block	Field type	Dose-mean lineal energy y_D [keV/ μm]
150 kVp X-rays	2.1-cm thick Pb	In-field	4.643 ± 0.066
		Out-of-field	4.687 ± 0.086
225 kVp X-rays	5.0-cm thick Pb	In-field	$4.393 \pm 0.007^{\text{a}}$
		Out-of-field	$4.769 \pm 0.044^{\text{a}}$

^a The values for 225 kVp X-rays were obtained from the previous report (Matsuya *et al* 2019).

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