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Raman spectroscopy of lymphocytes for the identification of prostate cancer patients with late radiation toxicity following radiotherapy

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Abstract

The success of radiotherapy in tumour control depends on the total dose given. However, the tolerance of the normal tissues surrounding the tumour limits this dose. It is not known why some patients develop radiation toxicity and, currently, it is not possible to predict before treatment which patients will experience adverse effects. Thus, there is an unmet clinical need for a new test to identify patients at risk of radiation toxicity. Here, we report a new approach based on Raman spectroscopy. Blood samples were collected from 42 patients who had undergone radiotherapy for prostate cancer and had shown either severe or no/minimal late radiation toxicity in follow up. Radiation response was assessed following in vitro irradiation using Raman spectroscopy in addition to the G2 chromosomal radiosensitivity assay and the γH2AX DNA damage assay. A Partial Least Squares Discriminant Analysis model was developed to classify patients using known radiation toxicity scores. A sensitivity of 95%, specificity of 92% and overall accuracy of 93% was
achieved. In the future, this technology may have potential to lead to individualised patient radiotherapy by identifying which patients are at risk of radiation toxicity.

Keywords
Raman spectroscopy; prostate cancer; radiotherapy; radiation toxicity; adverse effects; lymphocytes

1 Introduction

Despite recent technological advances to conform the dose of radiation to the tumour, normal tissue is always irradiated during radiotherapy and this can lead to the development of acute or late toxicity for the patient (1). Late toxicity can continue years after completion of radiotherapy and may be irreversible resulting in decreased quality of life. Known causes of radiation toxicity include dose volume parameters, co-morbidities such as diabetes, intrinsic radiosensitivity and concurrent chemotherapy (2). However, there is a large patient to patient variability in response which is intrinsic to the patient. To date, no markers of tumour response to radiotherapy or predictors of normal tissue toxicity are in routine clinical use.

Research on predictive assays of normal tissue radiosensitivity has been ongoing for several years and has so far yielded conflicting results. The early colony forming assays involving in vitro irradiation of fibroblasts from skin biopsies showed that in vitro cellular radiosensitivity was indicative of in vivo normal tissue response (3,4). However, establishing fibroblast cultures in vitro is time consuming and costly and peripheral blood lymphocytes have been shown to be a good alternative for measuring individual radiosensitivity (5). Survival assays
with lymphocytes from peripheral blood samples have been shown to predict the probability of developing late normal tissue damage (6,7). However, these assays are slow, labour intensive and require significant technical expertise making them unsuitable for routine clinical use. Assays involving in vitro irradiation of peripheral blood lymphocytes and subsequent assessment of DNA or chromosome damage have also been employed for determining individual radiosensitivity. Studies measuring chromosomal aberrations have shown correlation between in vitro cellular radiosensitivity and in vivo normal tissue response (8–10). In contrast, other studies have found no such correlations (11,12). Using the γH2AX assay, residual DNA damage has been shown to correlate with late normal tissue response (13–15), although other studies have not shown such a clear relationship (11,16–18). The radiation-induced lymphocyte apoptosis assay (RILA) has shown good evidence of an inverse correlation between RILA and radiation toxicity (15,19–22) but again other studies did not show a clear correlation (11,18). In addition, these lymphocyte assays may not be easy to translate to routine clinical use due to intrinsic variability and labour intensive protocols.

Initial results from genome wide association studies (GWAS) show evidence of association between common genetic variants and a cancer patient’s risk of developing radiation toxicity (23,24),(25). However, genomic assays are labour intensive and expensive.

The present study takes a new approach based on optical spectroscopy which has advantages over the lymphocyte and genomic assays in terms of minimal sample preparation, speed and cost. Raman spectroscopy is based on inelastic scattering of light and can provide a rapid, label free, non-destructive measurement of the biochemical fingerprint of a cell. Over the past 15 years, there have been numerous studies showing the potential of Raman spectroscopy for disease screening and diagnosis and very promising
results using cells, tissues and biofluids (26). Recent studies have shown the potential of Raman spectroscopy to characterise radiation response of normal and tumour cells irradiated \textit{in vitro} (27–31) and of tumour tissue irradiated \textit{in vivo} (32).

The aim of the present study was to evaluate Raman spectroscopy as an assay for identification of late normal tissue toxicity by investigating spectral differences in lymphocytes from prostate cancer patients with severe late toxicity (grade 2+) and those with no/minimal late toxicity (grade 0-1). \textit{In vitro} cellular radiosensitivity was also assessed in parallel using the G2 radiosensitivity assay, which measures the number of chromosomal aberrations induced by radiation in lymphocytes in the G2 phase of the cell cycle and the $\gamma$H2AX assay, which measures DNA damage.
2 Materials and Methods

2.1 Patients

Two hundred and fifty two patients were enrolled on the Cancer Trials Ireland (formerly the All-Ireland Co-operative Oncology Research Group ICORG) 08-17 study, ICTRP ID: NCT00951535; A Prospective Phase II Dose Escalation Study Using IMRT for High Risk N0 M0 Prostate Cancer, a prospective, phase II non-randomised controlled clinical study where the primary endpoint is to determine if dose escalation up to 81 Gy using IMRT for high risk localised prostate cancer can provide prostate specific antigen (PSA) relapse-free survival similar to that previously reported (33). Prostate specific antigen (PSA) levels and toxicity, using the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) grading system, have been recorded prior to treatment, during treatment and at follow up. Patients are followed up at 2 months post radiation therapy (RT), 8 months post RT and at 6 monthly intervals until Year 9. Radiotherapy volume/dose metrics have been recorded for planning target volumes (PTVs) and organs at risk (OARs) together with details on duration of treatment, treatment breaks etc. All patients were prescribed either 6 months or 3 years of neo-adjuvant / adjuvant hormone therapy.

Of the 147 patients who had been followed up 18-24 months post RT, 56 patients were identified with grade 2+ genitourinary (GU) or gastrointestinal (GI) late radiation toxicity and of these, 25 patients consented to participate and provide a blood sample. These patients were matched with 17 patients who showed no/minimal (grade 0-1) late radiation toxicity who also had been followed up for at least 18 months post RT. Patients were matched as far as possible based on age, tumour stage, Gleason score, PSA level and RT volume/dose metrics. Clinical details of both patient groups are shown in Table 1. All patient data was anonymised to maintain patient confidentiality.
The translational research study was approved by the Research Ethics Committee and all research was performed in accordance with relevant guidelines and regulations. Informed consent was obtained from all participants. Fresh whole blood, approx. 20 ml, was drawn into Li-heparin tubes and were coded before being transferred to the laboratory. Blood samples were cultured within 24 hours of extraction. Figure 1 shows an overview of the patient identification, sample processing, *in vitro* irradiation and subsequent assays.

2.2 Whole blood culture

Whole blood cultures were prepared by adding 2ml heparinised blood to 18ml RPMI with 12.5% (v/v) FBS and 2mM L-glutamine (Sigma Aldrich) supplemented with 2.5% (v/v) phytohaemagglutinin (PAA Laboratories). A total of 6 T25 flasks were prepared for each donor and incubated in an upright position for 72 hours at 37°C, 5% CO$_2$. Twenty-four hours before irradiation, 15ml media was replaced with fresh pre-warmed media supplemented with 2.5% (v/v) phytohaemagglutinin (PAA Laboratories).

2.3 PBMC culture

Peripheral blood mononuclear cells (PBMC) were isolated by adding 6ml Dulbecco's modified phosphate buffered saline (DPBS; Sigma Aldrich) to 6ml heparinised blood and overlaying onto 15ml Histopaque (Sigma Aldrich). After centrifugation at 400 g for 30 min, the PBMC layer was removed and cells were pelleted and resuspended in RPMI with 12.5% (v/v) FBS and 2mM L-glutamine (Sigma Aldrich) supplemented with 2.5% (v/v) phytohaemagglutinin (PAA Laboratories). One ml of cell suspension was transferred to each of 3 T25 flasks per donor containing 4ml of full media. Flasks were incubated for 72 hours at
37°C, 5% CO₂ lying flat to allow separation of lymphocytes and monocytes by plastic adherence.

2.4 In vitro irradiation

Cultures were irradiated after 72 hours using a 6MV photon beam produced by an Elekta Precise linear accelerator (LINAC), operating at a nominal dose rate of 6Gy/min. Doses of 0.05 and 0.5 Gy were used as low dose irradiation is routinely used in in vitro cellular radiosensitivity assays (12,14,15). In order to achieve a uniform irradiation of flasks, a 30x35cm² field was used and the flasks were positioned 10cm deep in a water equivalent phantom 90cm from the source. The calculated doses were verified using calibrated Gafchromic EBT3 film (Ashland Inc., NJ), scanned using an Epson Expression 10000XL scanner (35–37). The film was analysed using FilmQA Pro (Ashland Inc.).

2.5 G2 Chromosomal Radiosensitivity Assay

Following irradiation, for the G2 assay, whole blood cultures were incubated with 0.2μg/ml colcemid at 37°C for 1h to arrest cells in metaphase. Ice cold 0.075M KCl (Sigma Aldrich) was added to lyse the red blood cells and after 20 min incubation on ice, to prevent chromatid damage repair, the sample was centrifuged at 300 g retaining the white blood cell pellet with chromosomes. The cells were fixed with 3:1 methanol (Sigma Aldrich):acetic acid (AppliChem GmbH). Slides were prepared by pre-cleaning in methanol (Sigma Aldrich) 24 hrs prior to use and then briefly washed and stored in deionized water. Two to three drops of the cell suspension were dropped onto the slides to break open the cell membrane and release the chromosomes in metaphase and were heat fixed. Slides were stained using a 3% Giemsa solution (Sigma Aldrich) prepared in a pH 6.8 buffer (VWR) for 20 min. Slides
were washed in deionised water and left to dry before being mounted in Coverquick (VWR). The % total number of sister chromatid aberrations was recorded microscopically from 50 cells per slide to generate a G2 chromosomal radiosensitivity score. Chromatid gaps and breaks constituted the majority of aberrations scored, but terminal deletions, chromatid minutes and rare chromatid exchanges were also recorded. Radiation-induced G2 scores for each donor were calculated by subtracting spontaneous aberrations on control unirradiated samples for that same donor. A G2 chromosomal radiosensitivity threshold of 132 aberrations/100 metaphases from a previous parallel study at our laboratory calculated as the 90% percentile of G2 scores in healthy donor samples at 0.5Gy (38) was used as the cut-off for G2 radiosensitivity in this study.

2.6 γH2AX DNA Damage Assay

For the γH2AX assay, PBMC cultures were incubated at 37°C for 1h post-irradiation, followed by fixation in 2% paraformaldehyde. Fixed cells were permeabilised in 0.25% (v/v) Triton X-100 (VWR) and blocked in 2% (w/v) bovine serum albumin (Sigma-Aldrich). After blocking, the cells were resuspended in primary antibody solution (Anti-phospho-histone H2AX; Merck Millipore) followed by washing before adding secondary antibody solution (Alexa Fluor 488; Invitrogen). The mean fluorescence signal intensity was measured using an Accuri C6 (BD Biosciences) flow cytometer. A minimum of 10,000 events per sample was recorded. Debris and cell aggregates were removed from the analysis using forward and side scatter characteristics.

2.7 Raman spectral analysis
For Raman spectroscopy, PBMC cultures were incubated at 37°C for 1h post-irradiation, followed by fixation in 2% paraformaldehyde (AppliChem GmbH). Fixed cells were drop cast onto calcium fluoride (CaF$_2$) slides followed by washing in deionised H$_2$O. The samples were allowed to dry prior to Raman spectroscopic measurements. Raman spectroscopy of lymphocytes was performed as described in Maguire et al (29). Briefly, Raman spectroscopy was performed using a Labram HR800 system (Horiba UK Ltd), equipped with a 660nm laser. A total of 50 spectra from each of the unirradiated and irradiated samples were collected from each patient. Spectra were recorded with a 20s integration time and averaged over three integrations. Each spectrum was recorded using a 4x4µm raster scan of the centre of each cell. Multiple calibration spectra of 1,4-Bis(2-methylstyryl)benzene (Sigma Aldrich) were recorded along with each sample acquisition. All spectra were subsequently wavenumber calibrated using in-house developed procedures in Matlab v.9.3 (Mathworks Inc., Natick, MA). The instrument response correction was performed using the spectrum of NIST Standard Reference Material (SRM) no.2245 (39). Baseline correction was performed using a rubberband baseline subtraction, and all spectra were standardized before analysis. Inter-sample averaging of spectra within each patient sample was conducted to produce approximately 5 representative spectra per patient within each class. This had the effect of both improving the signal to noise ratio and time to execution of the models. All analysis was conducted in Matlab with the PLS Toolbox v.8.0 (Eigenvector Research Inc.).

2.8 Data analysis

For the G2 assay and the γH2AX assay, an unpaired two-tailed t-test was used at a 0.05 significance level to determine if there was a statistically significant difference between the
two patient groups. Similarly, for the Raman difference spectra, a two-tailed t-test by wavenumber was used.

In this study, classification of spectra from both grade 0-1 and grade 2+ patients was performed using a partial-least squares discriminant analysis (PLSDA) approach (40). Briefly, PLSDA is a discriminant analysis approach based on partial least squares regression, where the y-variable (the regression target) is encoded as the discrete spectral class (in this case the patient’s radiation toxicity).

PLSDA models were built independently from spectral datasets of lymphocytes irradiated to 0Gy, 0.05Gy and 0.5Gy. To investigate whether the Raman spectra at any dose contained differentiating signals allowing the classification of patients according to radiation toxicity, PLSDA models were trained with spectra at that dose using 90% of the patients from each toxicity class, and validated with the remaining 10% of patients. Patients were randomized between the training and validation sets automatically at the start of each algorithm development phase. A total of 20 independent models were constructed with an increasing number of latent variables, with the classification accuracy, sensitivity and specificity evaluated at the point in each model at which the sensitivity and specificity were optimized. The performances quoted here are the average performance for each independently cross-validated model.
3 Results

3.1 G2 Chromosomal Radiosensitivity of Grade 0-1 and Grade 2+ patients

For both the grade 0-1 and the grade 2+ patients, the number of spontaneous aberrations was very low (mean 7.2, range 0-20, for grade 0-1 patients and mean 9.4, range 0-28, for grade 2+ patients). Figure 2 shows the radiation induced G2 scores for the grade 0-1 and grade 2+ patients. Significant inter-individual variation in G2 radiosensitivity was observed in both groups, with more variability in the grade 2+ group. The mean G2 score was 113 (range 32-188) for grade 0-1 patients and 152 (range 60-268) for grade 2+ patients but there was no statistically significant difference in G2 score between the two groups. The grade 2+ group however, showed a very different distribution to the grade 0-1 group with a bimodal distribution (p=0.0001).

3.2 γH2AX fluorescence of lymphocytes from Grade 0-1 and Grade 2+ patients

Figure 3 shows the increase in γH2AX fluorescence 1 hour after in vitro irradiation for the grade 0-1 and grade 2+ patients. Both groups showed an increase in γH2AX fluorescence at each dose with a more pronounced increase in the grade 2+ group at both 0.05Gy and 0.5Gy. However, the difference was not statistically significant due to the high level of inter-individual variation particularly in the grade 2+ group.

3.3 Raman spectral analysis of lymphocytes from Grade 0-1 and Grade 2+ patients

Radiation response of lymphocytes from Grade 0-1 and Grade 2+ patients

To investigate the radiation response of the lymphocytes from grade 0-1 and grade 2+ patients to in vitro irradiation, the difference spectra of unirradiated and irradiated lymphocytes were calculated for each patient group separately. Figure 4 shows the mean
spectra of unirradiated and irradiated lymphocytes from grade 0-1 and grade 2+ patients and the difference spectra of unirradiated and irradiated lymphocytes for each patient group. The shaded regions show where the spectra of the irradiated lymphocytes were found to be significantly different to the unirradiated lymphocytes (p<0.05).

A summary of the band changes and assignments is shown in table 2. In general, changes in nucleic acids, proteins and lipids were observed after irradiation to 0.05 and 0.5Gy in lymphocytes from grade 0-1 and grade 2+ patients with more significant differences observed between unirradiated and irradiated lymphocytes from grade 2+ patients.

3.4 Spectral difference between lymphocytes from Grade 0-1 and Grade 2+ patients

Next, the spectral difference between lymphocytes from grade 0-1 and grade 2+ patients was investigated to determine if intrinsic biochemical differences could be identified. Figure 5 shows mean and difference spectra of unirradiated lymphocytes from the grade 0-1 and grade 2+ patients. The shaded regions represent where the difference was found to be statistically significant (p<0.05). A summary of the band changes and assignments is shown in Table 3. Increases in bands associated with some vibrations of DNA, RNA, proteins, carbohydrates and carotenoids were observed in grade 2+ patients when compared to grade 0-1 patients. Decreases in bands associated with saccharides, lipids, proteins and other vibrations associated with DNA and RNA were observed in grade 2+ patients when compared to grade 0-1 patients.

3.5 Classification of Grade 0-1 and Grade 2+ patient groups

Classifications of lymphocyte spectra from grade 0-1 and grade 2+ patients were performed to investigate if the two patient groups could be discriminated. PLSDA models were
developed as described earlier. Independent models were built using spectra of unirradiated and irradiated lymphocytes. The resulting accuracies, sensitivities and specificities are provided in Table 4 and are calculated for the cross-validated PLSDA model. An example of the cross-validation performance of the PLSDA model is shown in Supplementary Figure S1.

The optimised PLSDA models performed relatively well for the unirradiated lymphocytes with an accuracy, sensitivity and specificity of 0.93, 0.95 and 0.92, respectively. Poorer performance was achieved for the irradiated lymphocytes. While the model complexity (indicated by the number of latent variables selected by the cross-validated model) is relatively high for the models at 0Gy, the generalizability of these models to unseen data, ie. data that has not been used for model development, reinforces the view that the models are being developed on real and consistent Raman signals observed in the data. Supplementary Figure S2 depicts an exemplary receiver operator characteristic which was calculated for the models on spectra at 0Gy. An additional analysis was also carried out whereby the class is randomly assigned to the spectra input to the model at training, as described by Westerhuis et al (41). The same training and testing methodology was then used and the results are depicted in Figure S3, where sensitivity and specificity average to 48% across the LV scale. This demonstrates that while the models reported here are relatively complex, a consistent and generalisable signal is identified and learnt by our PLSDA models.
4 Discussion

An increased mean G2 score was observed in the grade 2+ patients compared to the grade 0-1 patients but this was not statistically significant due to inter-individual variability. Previous studies by Finnon et al (11) for breast cancer patients and Brzozowska et al (42) for prostate cancer patients also observed significant inter-individual variability in G2 radiosensitivity and no significant difference between patients with or without late adverse reactions. Our previous study established a radiosensitivity cut off value of 132 using the 90th percentile of the G2 scores of healthy individuals (38) which is in line with radiosensitivity cut off values that have been reported previously (43–49). Using this G2 cut off score of 132, only 27% of the grade 0-1 group compared to 60% of the grade 2+ group were above the radiosensitivity cut off value. Although it would have been more relevant to carry out the G2 assay on PBMC cultures for this study as PBMC cultures were used for the γH2AX assay and the Raman assay, it was decided to use whole blood cultures as these are routinely used for the G2 assay (43-49) but also because our previous study establishing the radiosensitivity cut off value of 132 (38) had used whole blood cultures. In addition, white blood cell counts would have been very informative, but unfortunately as only one of the blood samples were available for the study, not enough material remained for these additional tests.

An increase in γH2AX fluorescence was observed for both groups 1 hour following in vitro irradiation at each dose but again no statistically significant difference was found between the two groups due to the high level of inter-individual variability particularly in the grade 2+ group. Although previous studies have shown correlations between residual γH2AX
fluorescence (>6 hours post irradiation) and late normal tissue toxicity (13,14,50), γH2AX fluorescence was assessed at 1 hour post irradiation in the present study to allow direct comparison to the well established G2 assay. Inter-individual variability in the early damage response may explain the lack of correlation to late normal tissue toxicity, but other studies have failed to show correlations between residual γH2AX fluorescence and late normal tissue toxicity (11,16,42). Although the flow cytometry γH2AX assay used here is not as sensitive as assays based on manual or automated foci scoring by microscopy, positive correlations between γH2AX fluorescence and late normal tissue toxicity have been found using both microscopy (14,50) and flow cytometry assays (13).

The radiation response of the lymphocytes from grade 0-1 and grade 2+ groups was investigated. Similar responses in spectral regions associated with DNA, RNA, proteins and lipids were observed for the grade 0-1 and grade 2+ groups, correlating well with previous studies on normal and tumour cells irradiated in vitro (27–30) and relating to DNA damage and cellular response to that damage. Interestingly, more significant differences between unirradiated and irradiated lymphocytes were observed in the grade 2+ group. This may be due to a more pronounced change in spectral features following in vitro irradiation for the grade 2+ group. This correlates well with the results of the in vitro cellular radiosensitivity assays where more pronounced effects were observed in the grade 2+ compared to the grade 0-1 group. A previous study by Maguire et al (29) using lymphocytes from healthy donors showed inter individual variability in classification performance between unirradiated and irradiated lymphocytes with higher sensitivity and specificity for some donors suggesting more pronounced changes in spectral features of their lymphocytes following in vitro irradiation. In the present study, the more pronounced effects were
observed in the grade 2+ group, suggesting increased radiosensitivity compared to the grade 0-1 group.

Significant spectral differences associated with vibrations of DNA, RNA, carotenoids, carbohydrates, proteins, lipids and other cellular components were observed between the grade 0-1 and the grade 2+ groups. Classification of grade 0-1 versus grade 2+ groups yielded good accuracy using the PLSDA model. Significantly, a high classification accuracy was achieved for the unirradiated lymphocytes from each group. This suggests that in vitro irradiation may not be required for the future prediction of patient toxicity and that the intrinsic spectral phenotype of the patient may be sufficient to discriminate on radiation toxicity. Interestingly, the intrinsic spectral differences between patients with grade 0-1 and grade 2+ toxicity were related not only to DNA, which is normally assessed in in vitro radiosensitivity assays, but also to proteins and lipids.

These findings, however, would need to be confirmed in a larger patient population and would need to be further validated in an independent set of patients. In addition, as the present study analysed blood samples from patients collected >18 months after radiotherapy, a further ongoing study is analysing blood samples from patients’ pre-treatment.

In conclusion, this study presents, for the first time, the novel application of Raman spectroscopy to identify radiotherapy patients with late normal tissue toxicity. Spectral differences were identified between lymphocytes from patients with severe (grade 2+) late radiation toxicity and those with no/minimal (grade 0-1) late radiation toxicity which allowed the classification of patients with a high degree of accuracy.
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References


Figure Captions

**Figure 1** Schematic showing an overview of the patient identification, sample processing, in vitro irradiation, G2 assay, γH2AX assay and Raman assay.

**Figure 2** (A) Giemsa stained metaphase spread showing chromosomal aberrations such as chromatid break (ctb), chromatid gap (ctg) and chromatid minute (ctm), (B) Radiation induced G2 scores for grade 0-1 and grade 2+ patients. The error bars indicate the standard deviation.

**Figure 3** Relative increase in γH2AX fluorescence 1 hour after in vitro irradiation to (A) 0.05Gy and (B) 0.5Gy for grade 0-1 and grade 2+ patients. The error bars indicate the standard deviation.

**Figure 4** Lymphocyte response to 0.05Gy and 0.5Gy in (A) grade 0-1 and (B) grade 2+ patient groups. Top panels display the mean spectra (with a shaded region around each mean spectrum indicating the standard error on the mean for that class) and bottom panels display the difference between the spectrum of the unirradiated and irradiated cells. Shading within the bottom panel represents regions of the spectrum that were found to be significantly different (two-tailed unpaired t-test with p<0.05).

**Figure 5** (A) Mean spectra of unirradiated lymphocytes from grade 0-1 patients (black) and grade 2+ patients (red). The shaded region around each spectrum indicates the standard error on the mean for each class. (B) Difference spectrum of unirradiated lymphocytes from
grade 0-1 and grade 2+ patients. Shading represents regions of the spectrum that were found to be significantly different (two-tailed unpaired t-test with p<0.05).
Patients with high risk prostate cancer, n=252

Radiotherapy

18-24 months

Patients with follow up, n=147

Patients with grade 2+ late normal toxicity, n= 25 consented from total of n=56

Patients with grade 0-1 late normal toxicity, n=17 matched from total of n= 91

Patient blood samples

Whole blood cultures

PBMC cultures

In vitro irradiation

72 h

1 h

G2 assay

γH2AX assay

Raman assay

Figure 1
Figure 2
Figure 3
Figure 4
Figure 5