Gamma-H2AX biodosimetry for use in large scale radiation incidents: comparison of a rapid lyse/fix protocol with a routine method

Following a radiation incident, preliminary dose estimates made by γ-H2AX foci analysis can supplement the early triage of casualties based on clinical symptoms. Sample processing time is important when many individuals need to be rapidly assessed. A protocol was therefore developed for high sample throughput that requires less than 0.1 ml blood, thus enabling finger prick sampling. The technique combines red blood cell lysis and leukocyte fixation in one step on a 96 well plate, in contrast to the routine protocol, where lymphocytes are separated by Ficoll density gradient centrifugation with subsequent washing and fixation steps. The rapid lyse/fix method reduced the estimated sample processing time for 96 samples to about 4 h compared to 15 h using the routine protocol. However, scoring 20 cells in 96 samples prepared by the rapid protocol took longer than for the routine method (3.1 versus 1.5 h at zero dose; 7.0 versus 6.1 h for irradiated samples). Similar foci yields were scored for both protocols and reliable dose estimates were obtained for coded samples, with mean absolute differences from the actual doses of 0.26 and 0.27 Gy for the routine and lyse/fix method, respectively. The lyse/fix protocol can therefore facilitate high throughput processing for γ-H2AX biodosimetry for use in large scale radiation incidents, at the cost of somewhat longer foci scoring times.

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6   INTRODUCTION

7   The γ-H2AX assay has been shown to be a reliable and sensitive indicator of radiation-induced
8   DNA double-strand breaks (Rothkamm & Löbrich, 2003; Ivashkevich et al, 2012). In addition,
9   several *ex vivo* studies (Horn, Barnard & Rothkamm, 2011; Horn & Rothkamm, 2011l Roch-
10   Lefevre et al, 2010; Mandina et al, 2011), have shown the potential of the γ-H2AX assay as a
11   useful biodosimetry tool from hours to ~3 days post exposure. The γ-H2AX assay can produce
12   dose estimates within a few hours of receiving a blood sample (Rothkamm et al, 2013a; Ainsbury
13   et al, 2013) making it a good candidate for high through-put biodosimetry in the case of a mass
14   casualty event. One option has been to develop fully automated systems such as the RABIT
15   system (Garty et al, 2010; Turner et al, 2011) capable of processing several thousand samples a
16   day. A drawback of this approach is that it requires highly sophisticated robotic equipment and
17   tailored process control software, which may be unavailable to some laboratories. An alternative
18   option to increase through-put is to have an assistance network of laboratories, such as those
19   involved in the European funded MULTIBIODOSE and RENEB projects, and to reduce the
20   processing time required for the γ-H2AX assay using routinely available equipment.

21   Here a method is presented for the assessment of γ-H2AX foci for biological dosimetry, that uses
22   a lyse/fix buffer to lyse red blood cells and fix leukocytes in one step from small 'finger prick'
23   blood samples. Potentially, the lyse/fix method would be a faster and more scalable technique for
24   high sample through-put compared to the routine protocol used to process samples for γ-H2AX
25   foci scoring, which involves the isolation of lymphocytes from at least 2 mL of blood using Ficoll
26   density gradient centrifugation with subsequent washing and fixation steps.

27   MATERIALS AND METHODS

28   **Blood sampling and irradiation**
29   Heparinised venous blood was taken with written informed consent and the ethical approval of
30   the Berkshire research ethics committee (Ref 09/H0505/87) from a panel of healthy donors.
31   Whole blood was exposed to 0.5 or 1.0 Gy of 250 kVp x-rays (with Cu/Al filtration) at a dose
32   rate of 0.5 Gy / minute (min). Zero radiation dose control samples were also included. Following
33   irradiation, the blood was held at 37 °C for 0.5 or 1 hour to simulate *in vivo* repair. Samples were
34   then processed for the assessment of γ-H2AX foci by the routine histopaque or rapid lyse/fix
35   method. A record was made of the time taken for each step in the process. To test the suitability
36   of the lyse /fix method for biological dosimetry, samples were exposed to x-ray doses up to ~4
37   Gy and incubated for 2 hours at 37 °C. Coded samples were processed using both methods,
38   scored and used to produce estimates of dose. Positive and negative reference samples (0 and 1
39   Gy) were also included.

40   **Rapid lyse/fix method**
41   Aliquots of 90 µL whole blood were placed into a 96 deep well plate. To each well, 1x lyse/fix
42   buffer (BD Biosciences, UK) pre-warmed to 37 °C was added to give a dilution of 1:20,
43   thoroughly mixed and incubated at 37 °C for 14 min. After centrifugation at 250 g for 5 min the
44   supernatant was removed using an 8 channel aspirator. Following two washes in cold phosphate
45   buffered saline (PBS) the cell pellet was re-suspended in ~50 µL of PBS. An aliquot of 25 µL per
46   sample was spotted onto a teflon/silane coated 14-well slide (Tekdon Incorporated, USA) and the
47   cells were allowed to adhere for ~15 min. When the slides were almost dry they were placed in a
48   moist camber and incubated in 0.5% Triton-X (Sigma, UK) in PBS for 5 min. The slides were

49 then drained and incubated for 10 min in blocking solution (BS), containing 1% bovine serum
50 albumin (Sigma, UK) in PBS. Once any excess liquid had been removed, 100 µL of mouse γ-
51 H2AX antibody (BioLegend, UK), diluted 1:500 in BS, was applied to each slide. After
52 incubating the slides in the dark for 40 min at room temperature they were washed 3 times in BS.
53 Slides were then incubated in the dark with 100 µL of goat anti-mouse AlexaFlour 488 secondary
54 antibody (Abcam, UK), diluted 1:500 in BS, together with 0.2 µg/mL 4',6-diamidino-2-
55 phenylindole (DAPI) for 30 min at room temperature. Finally the slides were washed three times
56 in PBS and mounted in Vectashield anti-fade solution (Vector Laboratories, UK). A detailed
57 description of the lyse/fix protocol, sample requirements and materials can be found in
58 *Supplementary material: A Rapid Protocol for γ-H2AX Processing.*

59 **Routine histopaque method**
60 The histopaque method has been described in detail by Rothkamm *et al* (2013b). In brief, 2 mL
61 of whole blood was mixed 50:50 with PBS and layered onto 4 mL Histopaque 1077 (Sigma, UK).
62 After centrifugation at 1200 g for 5 min, the buffy coat layer was washed twice in PBS and the
63 cell pellet re-suspended in ~0.1 mL of PBS. 10 µL of the cell suspension was spotted onto a
64 multi-well slide. Cells were allowed to adhere to the slide for ~15 min and then fixed in 2%
65 formaldehyde in PBS for 5 min, followed by 5 min in 0.25% Triton-X100 + 0.1% glycine in
66 PBS. At this stage the blocking step and the immuno-staining was carried out in the same manner
67 as the lyse/fix method described above.

68 **Manual scoring of γ-H2AX foci**
69 All the slides were examined at x600 magnification using a Nikon Optiphot 2 fluorescence
70 microscope, equipped with separate filters for DAPI and fluorescein isothiocyanate (FITC).
71 Manual scoring was timed for three unirradiated and three 1 Gy/1 h incubated samples on slides
72 produced by both processing methods. A total of 50 lymphocytes were scored per sample and the
73 time taken to do this was recorded every ten cells. To ensure the lyse/fix processing itself did not
74 affect foci levels, samples irradiated at 0.5 Gy/repair time 30 minutes and prepared using both
75 protocols were used to assess the number of foci per cell seen in a panel of donors. 50
76 lymphocytes were scored in each of the reference samples and the foci numbers were used to
77 adjust the calibration coefficients and the associated standard errors (Rothkamm et al, 2013b) of
78 the laboratory's calibration curve (Horn, Barnard & Rothkamm, 2011). Whole body dose
79 estimates for the unknown samples were produced by scoring up to 50 lymphocytes or 200 foci
80 per sample.

81 **Automated scoring of γ-H2AX foci**
82 The performance of automated scoring was also assessed on samples processed by the two
83 methods. For each processing method and the two irradiation conditions, 10 fields of view were
84 scored automatically. Maximum projection images of seven z planes at 1 µm step size were
85 captured for γ-H2AX and DAPI staining (only one central plane imaged) using a x40 objective.
86 Foci analysis was performed with the TRI2 program, which contains batch processing
87 functionality and automatic sorting of images. The method used for foci counting and analysis
88 was the Compact Hough and Radial Map (CHARM) algorithm aimed at faint and ill-defined
89 shapes (Barber et al, 2007). The foci analysis software has been fully described in Rothkamm *et*
90 *al.* (2012).

91 RESULTS AND DISSCUSSION

92   The time taken to process 16 samples using the lyse/fix protocol and 8 samples by the histopaque
93   method is very similar, 179.5 min and 171 min respectively (Figure 1). The time for each step in
94   both protocols has been recorded and an estimate made of the timings for handling of 96 samples.
95   Scaling up the timings for preparing 96 samples by the lyse/fix or histopaque method gives an
96   increase in the estimated processing time of a factor of ~1.4 (256 min total) and ~ 2.5 (439 min
97   total) respectively. Most of the difference between the two protocols is the time taken to process
98   the samples through the first 3 steps. Provided that multi-channel pipettes and aspirators for
99   liquid handling are used, one person could complete the first three steps of the lyse/fix protocol
100 for 96 samples in 72 min. If one person were to process 96 samples by the histopaque method up
101 to and including step 3 this would take more than three times as long (226 min). In reality, given
102 the long processing time required for one person to get 96 samples to step 4 of the histopaque
103 protocol, which may adversely affect the blood separation or lower foci levels, either several
104 persons would need to work together or the samples be divided into batches. Assuming four
105 batches of 24 samples each, the histopaque processing time for 96 samples would be 880 minutes
106 (14.7 h) for one operator (Figure 1).

107 Usually in the routine histopaque protocol cell suspensions are spread onto individual slides, but
108 for a large number of samples this would be time consuming. For the purposes of this
109 comparison, cells prepared by both techniques were spotted onto 14-well slides. The time taken
110 to complete steps 5 to 11 would be similar for both protocols, 169 (lyse/fix) and 198 min
111 (histopaque). Multi-well slides also enable one person to carry out the protocol after lysis and
112 fixation, as the time between the addition of a solution to the first well and the last is not so great
113 as to adversely affect any step of the staining protocol. However, this is not the case for the
114 histopaque protocol, which requires cells to be fixed after they have been placed on the slide, and
115 this is a time critical step. Again, several persons would be needed to process all the slides
116 together; alternatively they could be stained in batches by one person.

117 Histopaque separation of whole blood produces a cell suspension containing lymphocytes. In
118 contrast, the lyse/fix protocol produces a cell suspension made up of lymphocytes and other white
119 cell types, which can make foci analysis more challenging. Figure 2 shows images of one field of
120 view of cells processed by the lyse/fix and histopaque methods from irradiated and unirradiated
121 samples. By comparing the images it can be seen that the lyse/fix method gives fewer
122 lymphocytes per field of view (i.e. round cells) and more debris. In addition the foci are not as
123 clearly visible as in samples processed by the histopaque method. Despite differences in the
124 appearance of samples produced by the two methods, manual scoring produced similar foci
125 yields in lymphocytes from a panel of donors exposed to 0.5 Gy x-rays, as shown in Figure 3.
126 Samples processed by the histopaque and lyse/fix method gave similar average foci counts of
127 6.83 ± 0.80 and 7.12 ± 0.51 respectively. These values are consistent with previous studies (Horn,
128 Barnard & Rothkamm, 2011; Chua et al, 2011; Rothkamm et al, 2007).

129 In order to estimate the time required to analyse 96 samples, three slides for each processing
130 method and dose point were scored. For each slide the time taken to score a batch of 10 cells was
131 recorded and then repeated a further 4 times. The average time taken to score each batch of 10
132 cells from the three slides was then used to estimate the time it would take one person to score 20
133 cells from 96 samples. Previous work has demonstrated that scoring 20 cells can produce dose
134 estimates that will reliably place samples into the correct exposure categories required for triage
135 (Rothkamm et al, 2013a). As expected the mean time taken to score a 0 Gy sample is less than for
136 a 1 Gy sample at 1 h post exposure for both preparation methods. However, for the 0 Gy sample
137 the estimated time taken to score 20 cells in 96 samples prepared by the lyse/fix protocol was

138   about twice as long as for those produced by the histopaque method; 3.1 h compared to 1.5 h. At
139   the higher dose of 1 Gy the difference in scoring time between the two processing methods was
140   not as great, (7.0 h compared to 6.1 h).

141   Simply comparing the lyse/fix protocol with the histopaque method for processing and scoring all
142   96 samples suggests the time taken would be similar for both irradiation conditions, 7.4 h versus
143   8.8 h (unirradiated) and 11.3 h versus 14.4 h (irradiated) respectively. However, the histopaque
144   protocol requires at least two people to achieve this compared to one for the lyse/fix method. A
145   more realistic comparison involves the 96 samples being split into 4 batches for the histopaque
146   method to be carried out by one person. When this is done the estimated time taken to process
147   and score 96 samples by the histopaque method is 16.2 h (unirradiated) and 20.8 h (irradiated).
148   This is approximately twice the time required for sample processing and scoring using the
149   lyse/fix protocol. Furthermore, reagent costs for the histopaque method (~£116/96 samples) are
150   twice as high as those for the lyse/fix protocol (~£57/96 samples).

151   The more challenging analysis procedure required for samples produced by the lyse/fix method
152   may be aided by an automated scoring approach. Image analysis software using a form factor
153   parameter would allow the positive discrimination of round lymphocyte nuclei, thereby enriching
154   their number in the analysed cell population (Valente et al, 2011). Here, TRI2 software was used
155   to identify round cell nuclei and score foci automatically (Rothkamm et al, 2012). Figure 4 shows
156   the automated scoring results of 10 fields of view for each processing method and the two
157   irradiation conditions. Automated foci counts are comparable between methods, although ten
158   times more scorable cells per field of view are seen with the histopaque protocol. The lyse/fix
159   protocol produced 3 false negative fields out of 10 compared to 1 for the histopaque method,
160   likely caused by high background fluorescence due to excessive debris in lyse/fix samples. These
161   results suggest that if automated scoring were to be used to analyse samples, at least 2
162   (histopaque) and 3 (lyse/fix) fields of view need to be scored and results checked to ensure
163   consistency. Further optimisation of the software may reduce the number of false negative fields.

164   To determine whether the rapid lyse/fix protocol can still provide dose estimates with similar
165   accuracy as the routine histopaque protocol, an intercomparison exercise was performed using
166   uniformly X-irradiated, coded samples. Figure 5 shows that the dose estimates obtained for the
167   two different protocols correlated very well with each other. Mean absolute differences from the
168   actual doses were 0.26 and 0.27 Gy for the histopaque and lyse/fix protocols, respectively.

169   CONCLUSION

170   The lyse/fix protocol enables a high sample throughput, with an estimated processing time for 96
171   donors of just over 4 hours, using finger prick-sized quantities of blood. This can be achieved by
172   one person, without the need for highly sophisticated equipment. The manual microscope based
173   analysis of samples produced by the lyse/fix protocol is more challenging, which increases the
174   scoring time, but has no adverse effect on dose estimation. Overall the rapid lyse/fix method
175   allows one person to process and score 96 samples in about half the time taken using the routine
176   protocol.

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Figure 1

Sample processing times for the lyse/fix versus the routine histopaque method for different numbers of samples.

Steps 1 – 11: 1 = lyse/fix or isolation; 2 = washing; 3= transfer; 4 = adhere; 5 = permeabilise (and fixation for histopaque method); 6 = blocking; 7 = stain1; 8 = wash; 9 = stain2; 10 = wash; 11 = mount.
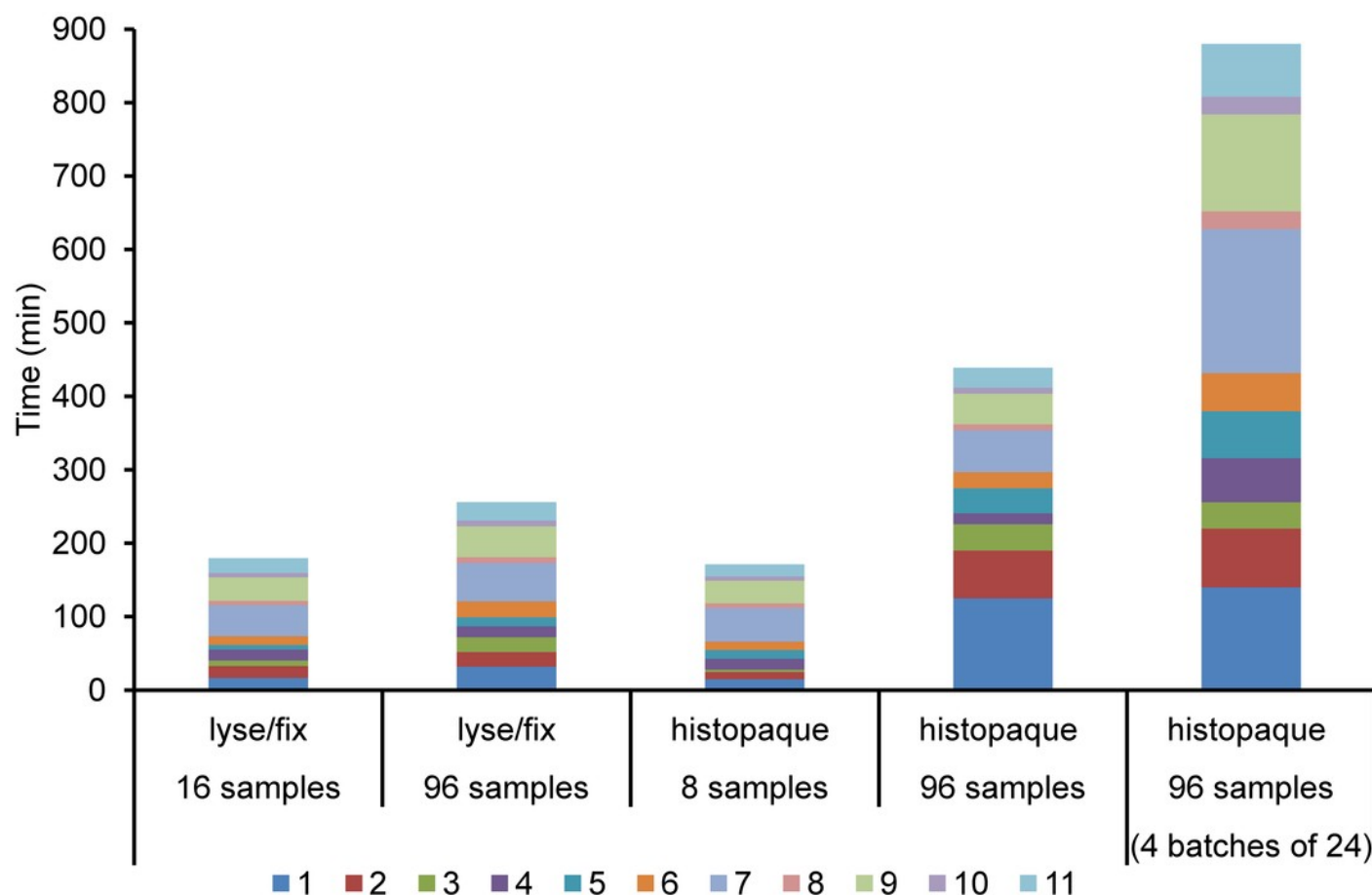
Figure 2

γ-H2AX foci in human blood leukocytes prepared with the lyse/fix or histopaque method.

Fluorescence microscopic maximum projection images (x40 objective) show γ-H2AX foci (green) and DNA counterstain (blue) following exposure to 0 or 1 Gy X-rays and incubation for 1 hour.
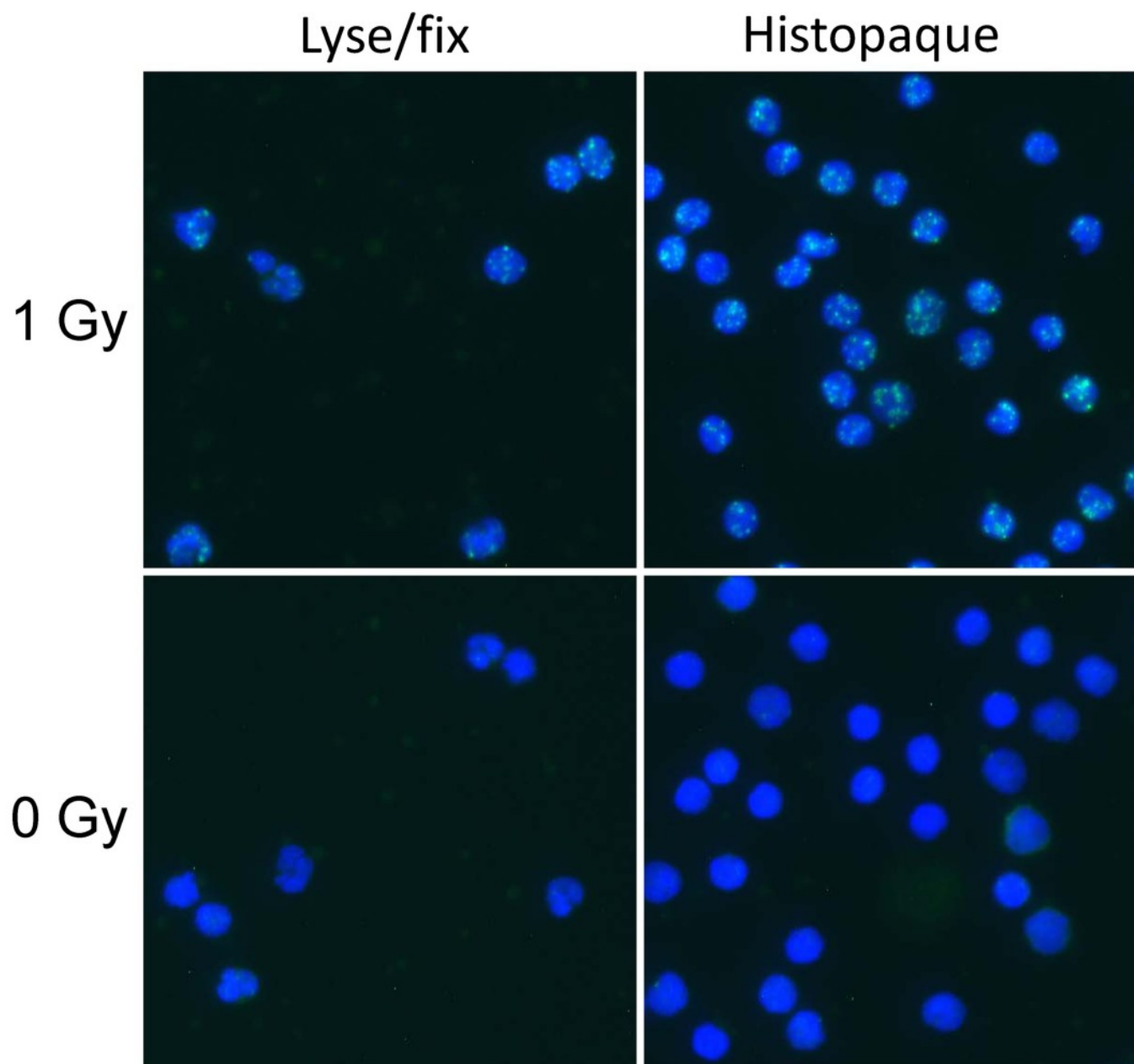
Figure 3

Comparison of manual γ-H2AX foci counts.

Different volunteer blood samples were processed by the lyse/fix or histopaque method following 0.5 Gy X-irradiation and 30 minutes incubation at 37 °C.
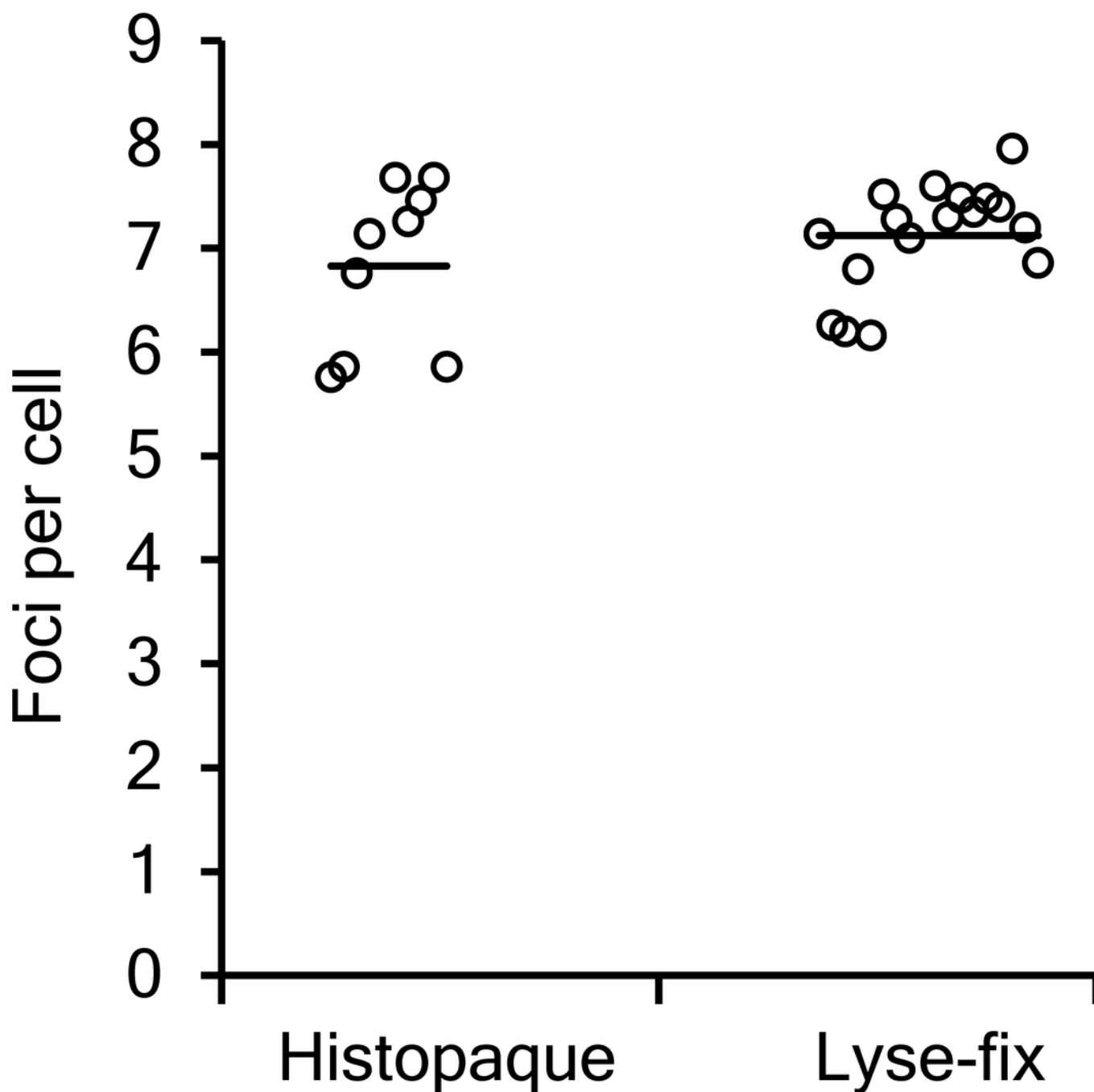
Figure 4

Comparison of automated γ-H2AX foci counts.

Ten fields of view were analysed using Tri2 software (Barber et al, 2007) for each processing method and two irradiation conditions (0 or 1 Gy plus 1 hour incubation at 37 °C). Open triangles: 0 Gy + lyse/fix; solid triangles: 1 Gy + lyse/fix; open squares: 0 Gy + histopaque; solid squares: 1 Gy + histopaque.
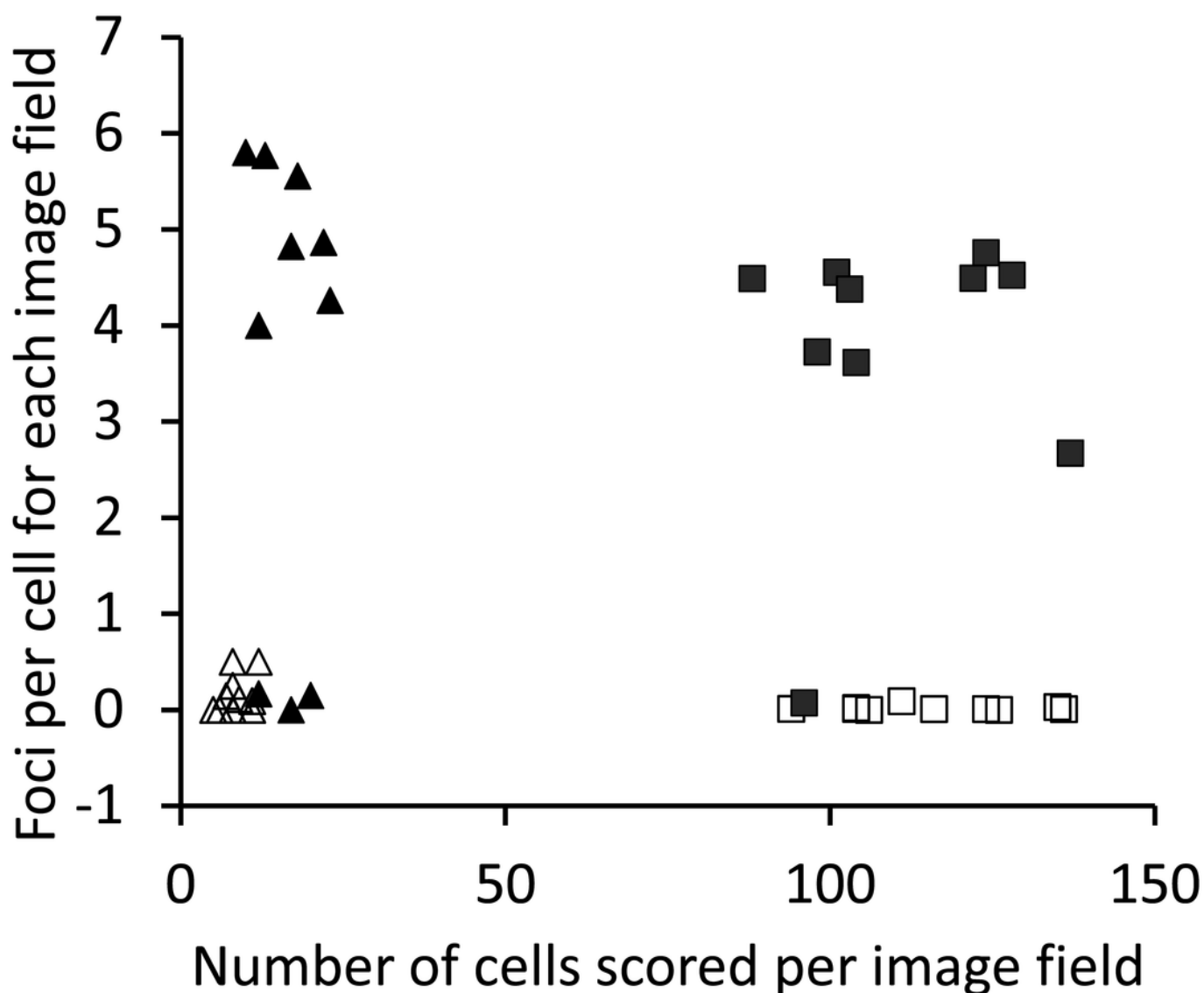
Figure 5

Comparison of whole body γ-H2AX dose estimates.

Foci yields were determined for X-irradiated and 2 hours incubated blood samples processed with the lyse/fix versus histopaque protocol. At least 50 cells or 200 foci were manually scored per coded sample. Foci yields were converted to dose estimates using calibration data from Horn et al, (2011). Actual doses were 0, 0.2, 0.6, 1.1, 1.2, 2.1 and 4.3 Gy. The line indicates the ideal 1:1 relationship.