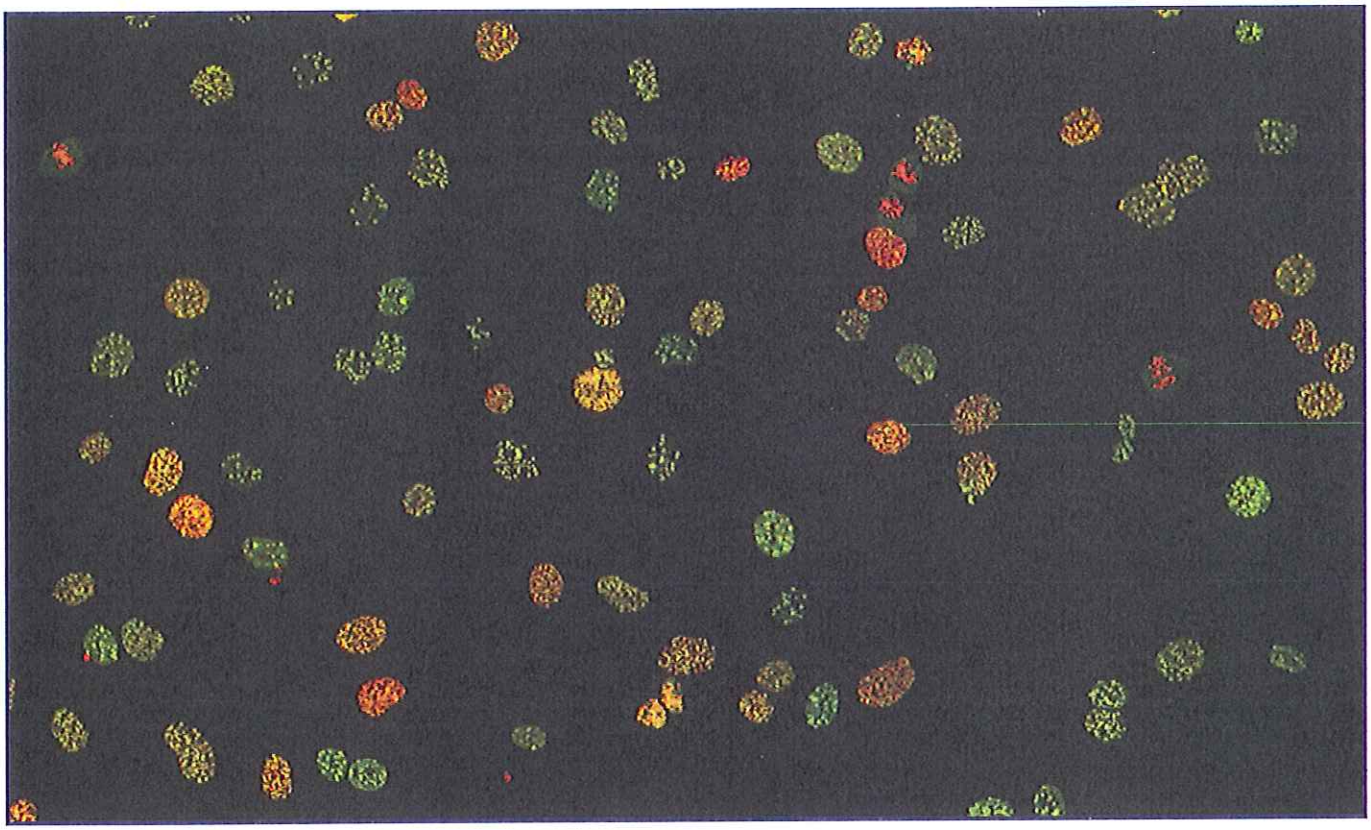
APPLICATION NOTE

BD Biosciences

DNA Damage and Repair - Quantification of Sub-Cellular Events Using Automated Confocal Imaging



Introduction

Oxidative stress, radiation and other external insults have been shown to damage DNA molecules in cells derived from organisms as diverse as bacteria, yeast, drosophila, rodents and man (Friedberg *et al.* 2005). The presence of DNA damage may lead to cell cycle checkpoint arrest to allow time for DNA repair processes to occur. If however, the system becomes overwhelmed or the DNA repair mechanisms are impaired, the cell either enters the apoptosis pathway or become cancerous due to the accumulation of mutations resulting from replication of damaged DNA. Therefore, a complete understanding of DNA repair mechanisms is of great interest in the study of cancer prevention and treatment. In addition, this process has been implicated in cellular senescence and aging (Sedelnikova *et al.* 2004).

Following induction of DNA double strand breaks, the specialized histone protein H2AX becomes phosphorylated and rapidly (within minutes) accumulates at the sites of DNA damage forming distinct foci (Paull *et al.* 2000).

H2AX foci formation is followed by recruitment of many other proteins involved in the DNA repair process including the p53 binding protein 53BP1 (Schulz *et al.*, 2000). Over long time periods (hours), the number of foci declines with the progression of DNA repair. Detection and quantification of foci development as an indicator of DNA damage and repair is of great interest to groups investigating these pathways (Kim *et al.* 2005). The assay is generally performed using coverslips and chamber slides and imaged on fluorescence microscopes (Sedelnikova *et al.* 2004). The demands for high spatial resolution and sophisticated image analysis, however, have hindered the transfer of this assay into a multi-well plate format and thus into the drug screening arena. Our goal was to establish image acquisition and analysis methods allowing quantification of foci development both over time and with increasing concentrations of H_2O_2, a known inducer of DNA damage.

BD Biosciences
15010 Broschart Road, Rockville, MD 20850
For more information visit **bdbiosciences.com/bioimaging**
toll free: 800.245.2614 (US)
301.340.7320 (Outside the US)

BD

Methods

Cell Culture

HT1080 cells (ATCC, CCL-121, human fibrosarcoma derived cell line) were grown in 37°C, 5%CO_2 humidified incubators according to the supplier's protocol. Cells were seeded at 10,000 cell/well into 96 well plates (Cat. No. 353219) and incubated overnight. H_2O_2 (Sigma, H-1009) was used to initiate DNA double strand breaks. Immediately prior to the experiment, H_2O_2 was diluted in pre-warmed media and dose-response curves were obtained by serial dilution. Cells were exposed to chemical insult for 10 minutes at room temperature after which they were washed and replenished with fresh media. To allow for DNA repair processes to occur, the cells were returned to the incubator and left for 30, 90, or 180 minutes. In some experiments, cells were left to recover for 16 hours. At those time points, cells were fixed for 10 minutes with pre-warmed formaldehyde, 3.7% in phosphate-buffered saline (PBS), washed with PBS and permeabilized with ice-cold 90% methanol (10 minutes). Cells were washed twice with PBS and blocked using 1% BSA (in PBS) for 30 minutes. For the detection of phosphorylated H2AX, the mouse monoclonal anti-phospho serine 139 H2AX antibody (Chemicon, 05-636) at 2 µg/mL in 1% BSA block was used. 53BP1 was detected using the anti-53BP1 rabbit polyclonal antibody (Novus Biologicals, NB 100-304) diluted as described for H2AX.

After 1h at room temperature, the primary antibody was removed and cells were incubated with Tween 20 (0.05% in PBS, 10 minutes). Two PBS washes prepared the cells for the addition of the secondary antibodies, 5 µg/mL Alexa Fluor® 555-conjugated anti-mouse IgG (Cat. No. A21424) for H2AX and 5 µg/mL Alexa Fluor® 488-conjugated anti-rabbit IgG (Cat. No. A11034) for 53BP1, respectively. The DNA intercalating dye Hoechst 33342 (Invitrogen, H3570) was added at this point at 2 µg/mL and removed with the secondary antibodies after 1h at room temperature (light protected). Cells were washed as described above and wells replenished with 200 µL of PBS. Plates were sealed with adhesive foil and imaged on the BD Pathway™ Bioimager. Analysis of the image data was performed using BD IPLab™ for Pathway (Cat. No. 340989) and plotted using BD™ Image Data Explorer (Cat. No. 341039).

Results

Treatment of HT1080 cells with H_2O_2 rapidly induced the formation of phosphorylated H2AX and 53BP1 foci (***Figure 1***). There is a marked increase in the number and intensity of foci in H_20_2 treated cells compared to control cells. The two foci shared a high degree of co-localization within nuclei as observed in the merged images.

Preliminary results had shown that the nuclear foci were not evenly distributed in the same focal plane (***Figure 2***). We used the ability of the BD Pathway Bioimager to acquire 9 sections separated by 1.5 µm steps in confocal mode. To achieve the required spatial resolution, we used a 40× (NA 0.90) objective. Using the novel "collapsed-stack" acquisition feature, images were then automatically projected into a single focal plane with all objects appearing in focus.

This allowed segmentation and identification of objects within the nucleus using a customized image analysis routine written in BD IPLab for Pathway. The collapsed stack images were compared to images acquired in non-confocal mode (***Figure 2*** shows part of an image containing a single nucleus). The analysis tool generated specific parameters such as foci count, foci area as percent nuclear area, average foci intensity and average foci size. On the cellular level, the response appeared highly heterogeneous and cells could be found that showed very few foci next to cells containing a large number of foci (***Figure 1***). We therefore sampled on average 120 cells/well in a 3×3 montage. Data was imported into BD Image Data Explorer where dose-response curves and Z' data were generated (***Figures 3 and 4***).

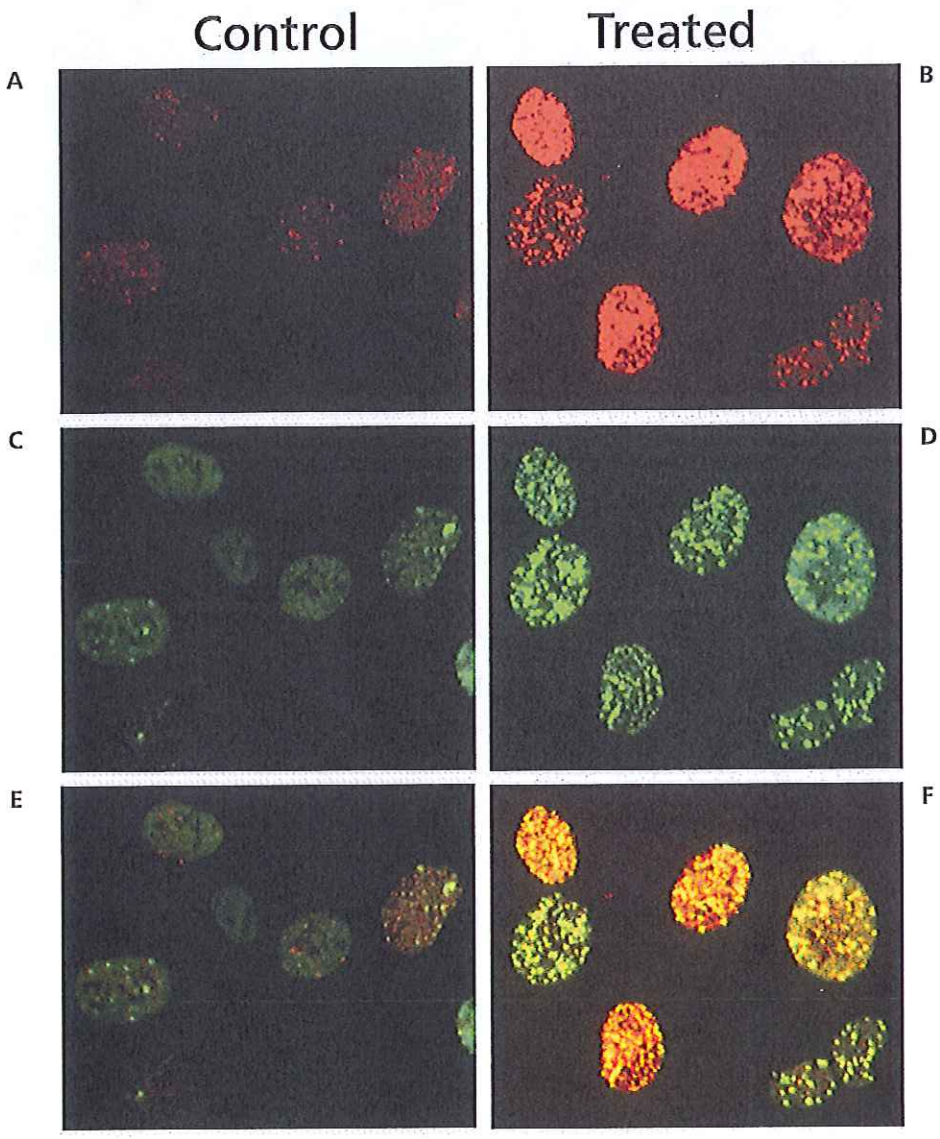


Figure 1. H_2O_2-induced H2AX and 53BP1 foci formation in HT1080 cells. Control (***panels A, C and E***) and H_2O_2 treated cells (250 µM, panels ***B, D and F***) are shown following a 30 min recovery time. The upper row (***A and B***) shows phosphorylated H2AX staining (red), the middle (***C and D***) shows 53BP1 staining (green) and the lower an overlay of both images (***E and F***). The images were acquired in confocal stack mode (see ***Figure 2***) and show the effect of H_2O_2 treatment on the number and intensity of foci at the locations of DNA damage. Note the high degree of spatial overlap between the 53BP1 and phosphorylated H2AX foci in panel F.

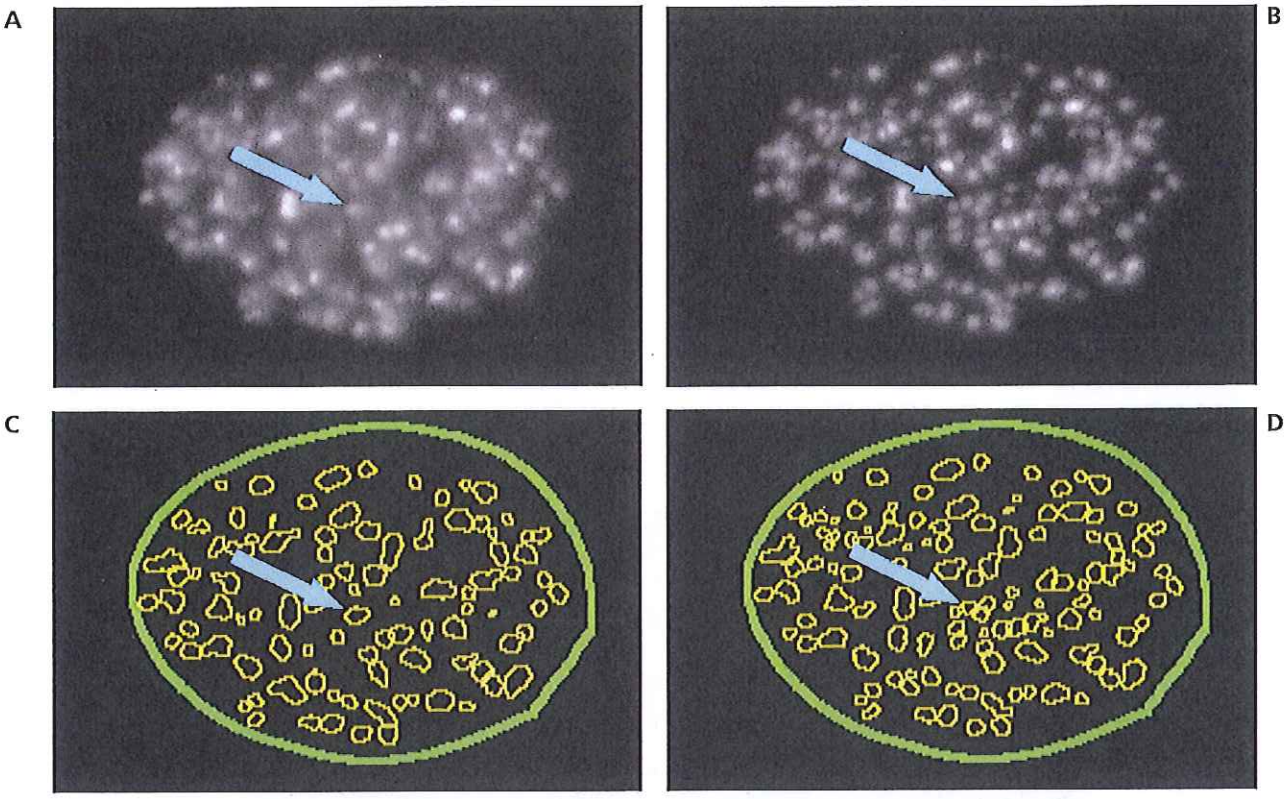


Figure 2. Comparison of images acquired in non-confocal or confocal mode. In non-confocal mode (***A***), the foci appeared fewer in number, larger and blurred (see arrows). The "collapsed-stack" feature of the BD Pathway Bioimager allowed acquisition of multiple Z positions and projection into a single image (***B***). The qualitative improvement seen with confocal over non-confocal imaging also translated into better assay performance (see ***Figure 3***). ***Panel C*** and ***D*** show the segmentation of the non-confocal **(A)** and confocal, collapsed stack ***(B)*** images, respectively. Note the greater number of foci (especially dim ones) identified by the image analysis algorithm (in the nucleus shown: 94 foci in non-confocal mode versus 118 in confocal collapsed stack mode corresponding to a 26% increase, panels C and D, respectively).

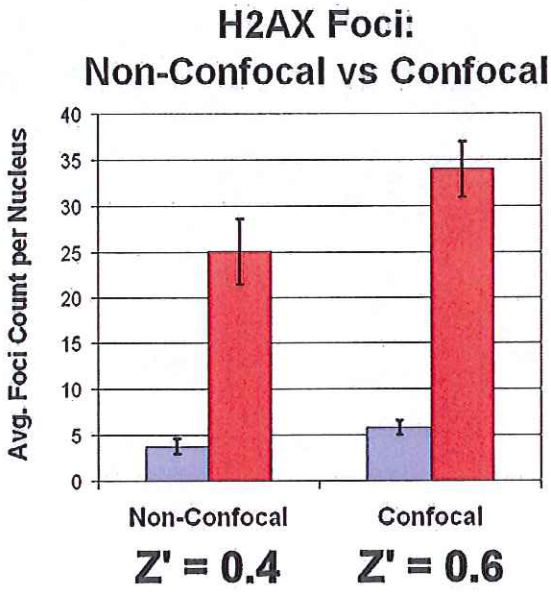


Figure 3. Comparison of assay performance under non-confocal and confocal collapsed stack acquisition conditions. The average number of H2AX foci per nucleus from control wells (blue bars) was compared to wells treated with 250 µM H_2O_2 (red bars). The data was averaged from 6 replicate wells from a 30 minute recovery time point. The statistical difference is expressed as the Z' and was 0.4 for non-confocal and 0.6 for confocal, collapsed stack.

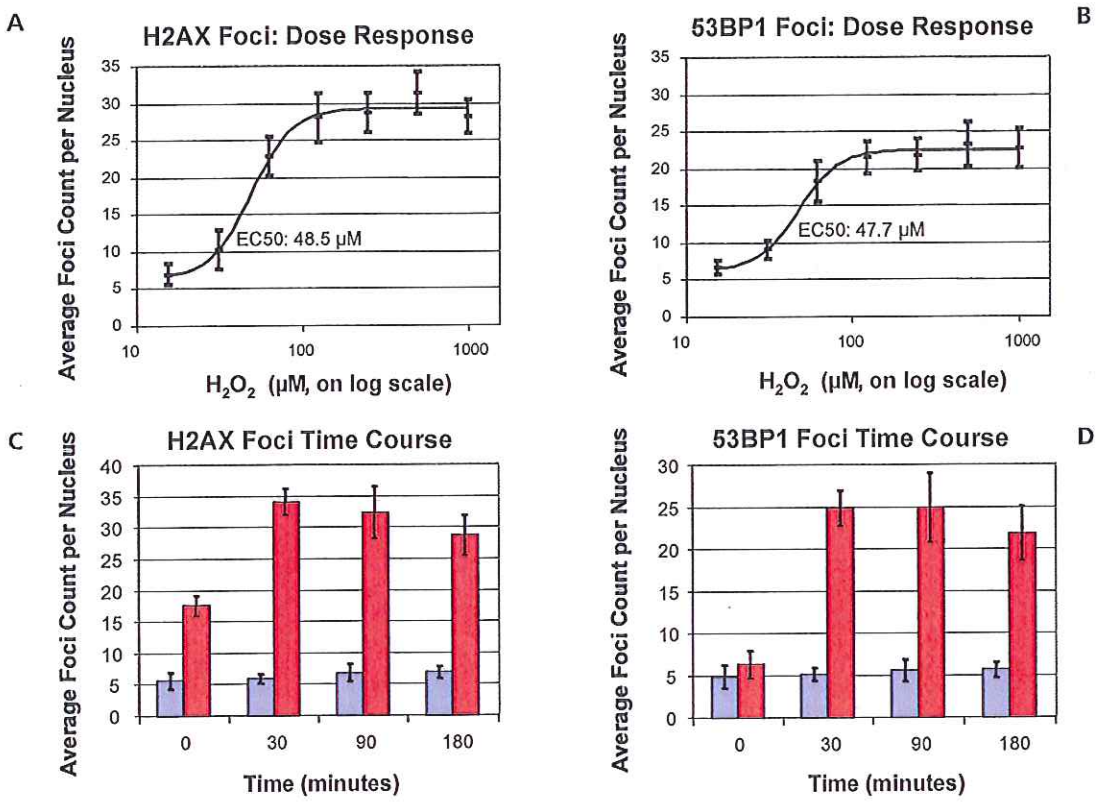


Figure 4. Dose-response relationship and time course of foci formation. Formation of H2AX foci (***panel A***) and 53BP1 (***panel B***) appears to plateau at 250 µM H_2O_2 for both proteins (shown at the 180 minute recovery time point). The dose response relationship generated an EC_{50} of 48.5 µM for H2AX and 47.7 µM for 53BP1.Time course of foci formation (***panels C and D***, blue bars = control, red bars = 250 µM H_2O_2 treated, n=6 wells) shows a distinct increase in the number of H2AX foci in cells allowed to recover for 30 minutes (***panel C***) whereas the increase of 53BP1 (***panel D***) lags behind and appears to peak between 30 and 90 minutes. The number of H2AX and 53BP1 foci slowly declined at longer time points (180 min). Overnight (16h) recovery further reduced the number of foci (data not shown) to approximately 2 fold of basal level.

Discussion

The ability to acquire confocal, collapsed stack images has allowed us to develop an automated assay relying on identification of small sub-nuclear features, namely foci of DNA damage and repair. The improved statistical performance of the assay in confocal, collapsed stack mode suggests that this assay indeed can be applied to a high-content screening environment.

We noted that even untreated cells contained some foci, suggesting that under basal conditions, some DNA double-strand breaks are present and repair occurs. Phosphorylated H2AX accumulation was seen immediately after chemical treatment confirming the rapid time course of the activation and recruitment of this protein to the sites of DNA damage. The subsequent accumulation of 53BP1 foci peaked between 30 and 90 minutes and is in agreement with published reports (Schultz *et al.* 2000). To our knowledge, the EC_{50} for H_2O_2-induced H2AX and 53BP1 foci formation has not been published and we believe that our assay can aid the characterization of other chemical agents and DNA repair proteins in a similar manner. The appearance of the 53BP1 foci in the same spatial location after 30 minutes confirms reports of rapid activation of the repair signaling cascade (Schultz *et al.* 2000).

Although we did not investigate the signaling properties between these two proteins, the assay is very suitable to investigate whether intermediate molecules are involved or whether 53BP1 is recruited by activated H2AX directly.

The comparatively straight-forward assay protocol using only 2 primary antibodies, 1 dye and a commercially available cell line allow this assay to be used in automated high-content screening applications where hands-off operation is required. The BD Pathway™ Bioimager with its confocal capability, white-light illumination and choice of 16 excitation filters allows development of multi-color assays and would even accommodate a 4th imaging channel. The complexity of change in the cellular phenotype in this assay mandate sophisticated and specialized image and data analysis algorithms. The combination of BD IPLab™ for Pathway and BD™ Image Data Explorer allows generation and analysis of high-content data in a drug screening environment.

References

1. Friedberg, E.C, Walker, G.C., Siede, *et al.* 2005 DNA Repair and Mutagenesis, ASM Press; 2nd edition *ISBN*: 1555813194
2. Kim, J-S, Krasieva, J_S, Kurumizaka, H, *et al.* 2005. Independent and sequential recruitment of NHEJ and HR factors to DNA damage sites in mammalian cells. *JCB,* 170, 341-347
3. Paull T.T., Rogakou E.P., Yamazaki V., Kirchgessner C.U., Gellert M., Bonner W.M..2000. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr Biol.* 10, 886-95.
4. Schultz, L.B, Chehab, N.H., Malikzay, A., Halazonetis, T.H., 2000. p53 Binding Protein 1 (53BP1) Is an Early Participant in the Cellular Response to DNA Double-Strand Breaks. *JCB,* 7, 1381-1390
5. Sedelnikova O.A., Horikawa I., Zimonjic D.B., Popescu N.C., Bonner W.M., Barrett J.C. 2004. Senescing human cells and ageing mice accumulate DNA lesions with unrepairable double-strand breaks. *Nature Cell Biology.* 6, 168-70.

Notes

Regional Offices

Asia Pacific
BD Singapore
Tel 65.6861.0633
Fax 65.6860.1593

Australia/New Zealand
Australia
Toll Free: 1800 656 100
Tel 61.2.8875.7000
Fax 61.2.8875.7200
bd_anz@bd.com

New Zealand
Toll Free: 0800 572.468
Tel 64.9.574.2468
Fax 64.9.574.2469
bd_anz@bd.com

Europe
Belgium
Tel 32.53.720.550
Fax 32.53.720.549
customer_service_bdbelgium@europe.bd.com

Canada
BD Biosciences
Toll Free 888.259.0187
Tel 905.542.8028
Fax 888.229.9918
canada@bd.com

Japan
Nippon Becton Dickinson
Toll Free 0120.8555.90
Tel 81.24.593.5405
Fax 81.24.593.5761

United States
BD Biosciences
Customer/Technical Service
Toll Free 877.232.8995
Bioimaging Systems
Fax 301.340.9775
Discovery Labware
Fax 978.901.7493
Immunocytometry Systems
Fax 800.325.9637
Pharmingen
Fax 800.325.9637
bdbiosciences.com

Local Offices and Distributors

Argentina/Paraguay/Uruguay
Toll Free: 0800.444.5523
Tel 54.11.4551.7100
Fax 54.11.4551.7400
crc_argentina@bd.com

Austria
Tel 43.1.706.36.60.20
Fax 43.1.706.36.60.11
customerservice.bdb.at@europe.bd.com

Brazil
Toll Free: 0800.771.7157
Tel 55.11.5185.9995
Fax 55.11.5185.9895
biosciences@bd.com.br

CCA (Caribbean/Central America)
Tel 52.55.5999.8318
Fax 52.55.5999.8396
caribbean_customers@bd.com
centralamerica_customers@bd.com

Chile
Tel 56.2 482.7800 x16
Fax 56.2 482.7802

China
Tel 8610.6418.1608
Fax 8610.6418.1610

Colombia
Tel 57.1.572.4060 x244 / 258
Fax 57.1.572.3650 / 4250

Denmark
Tel 45.43.43.45.66
Fax 45.43.43.41.66
bdbnordic@europe.bd.com

East Africa
Tel 254.2.341157
Fax 254.2.341161
bd@africaonline.co.ke

Eastern Europe
Tel 49.6221.305.161
Fax 49.6221.305.418
bdb.ema@europe.bd.com

Egypt
Tel 202.268.0181
Fax 202.266.7562

Finland
Tel 358.9.88.70.7832
Fax 358.9.88.70.7817
bdbnordic@europe.bd.com

France
Tel 33.4.76.68.36.09
Fax 33.4.76.68.35.06
customerservice.bdb.france@europe.bd.com

Germany
Tel 49.6221.305.551
Fax 49.6221.303.609
customerservice.bdb.de@europe.bd.com

Greece
Tel 30.1.940.77.41
Fax 30.1.940.77.40

Hong Kong
Tel 852.2575.8668
Fax 852.2803.5320

Hungary
Tel 36.1.345.7090
Fax 36.1.345.7093
bdb.ema@europe.bd.com

India
Tel 91.124.238.3566.70
Fax 91.124.238.3225

Indonesia
Tel 62.21.577.1920
Fax 62.21.577.1925

Italy
Tel 39.02.48.240.1
Fax 39.02.48.20.33.36

Korea
Tel 822.3404.3700
Fax 822.557.4048

Malaysia
Tel 603.7725.5517
Fax 603.7725.4772

Mexico
Toll Free: 01.800.236.2543
Tel 52.55.5999.8296
Fax 52.55.5999.8288
bioscienciasmexico@bd.com

Middle East
Tel 971.4.337.95.25
Fax 971.4.337.95.51
bdb.ema@europe.bd.com

The Netherlands
Tel 31.20.582.94.20
Fax 31.20.582.94.21
customer_service_bdholland@europe.bd.com

North Africa
Tel 33.4.76.68.35.03
Fax 33.4.76.68.35.44
bdbiosciences_maghreb@europe.bd.com

Norway
Tel 47.73.59.12.00
Fax 47.73.59.12.01
norge@europe.bd.com

Pakistan
Tel 92.42.5718051
Fax 92.42.5718056
bdb_pak@bd.com

Peru/Bolivia/Ecuador
Tel 51.1.712.0160
Fax 51.1.712.0165

Philippines
Tel 632.815.8981
Fax 632.815.6644

Poland
Tel 48.22.651.75.88
Fax 48.22.651.75.89
bdb.ema@europe.bd.com

Portugal
Enzifarma
Tel 351.21.421.93.30
Fax 351.21.421.93.39

South Africa
Tel 27.11.603.2620
Fax 27.11.804.0544 / 46
bdb.ema@europe.bd.com

Spain
Tel 34.902.27.17.27
Fax 34.91.848.81.04

Sweden
Tel 46.8.775.51.10
Fax 46.8.775.51.11
bdbnordic@europe.bd.com

Switzerland
Tel 41.61.485.22.22
Fax 41.61.485.22.00
customerservice.bdb.ch@europe.bd.com

Taiwan
Tel 8862.2722.5660
Fax 8862.2725.1768

Thailand
Tel 662.646.1800
Fax 662.646.1801

Turkey
Tel 90.212.328.2720
Fax 90.212.328.2730
bdb.ema@europe.bd.com

United Kingdom & Ireland
Tel 44.1865.78.16.88
Fax 44.1865.78.16.27
BDUK_CustomerService@europe.bd.com

Venezuela
Tel 58.212.241.3412 x248
Fax 58.212.241.7389

West Africa
Sobidis
Tel 225.20.33.40.32
Fax 225.20.33.40.28

BD Biosciences
10975 Torreyana Road
San Diego, CA 92121-1106
Toll free 877.232.8995
bdbiosciences.com

06-A790030-6A