High Throughput RNAi Screen for DNA Damage

Phosphorylated H2AX (Validated Assay)

RNA interference (RNAi) libraries combined with high throughput cell-based assays are an attractive strategy to identify novel mechanisms of drug sensitivity and resistance. For example, researches have reported a genome-wide RNAi screen that identified genes that sensitized non-small-cell lung cancer cells to paclitaxel (Whitehurst et al., Nature 446, 815-819, 2007). This study demonstrated the potential of the RNAi technology to identify novel targets important for mitotic progression in cancer cells. Blueshift Biotechnologies Inc. (BBI) has developed a powerful new screening platform, the **ImageXpress® Velos System**, with integrated image acquisition and analysis modules for fast data generation. In Application Note No. 8 we reported a fast and simple mitotic index assay where the effect of paclitaxel on mitotic cells was identified by the immunofluorescent detection of phosphorylated histone H3 and DNA content. Here we report on our development of a two-color assay for DNA damage based on the immunofluorescent detection of phosphorylated histone H2AX and DNA content. The phosphorylation of histone H2AX on serine 139 has been shown to be an early event and sensitive indicator of DNA double-strand breaks produced by drugs or ionizing radiation. We also show that this two-color DNA damage assay is a sensitive and fast automated process for RNAi screens and that this method may prove useful for target discovery efforts. A cartoon of the assay setup is shown in **Figure 1.**

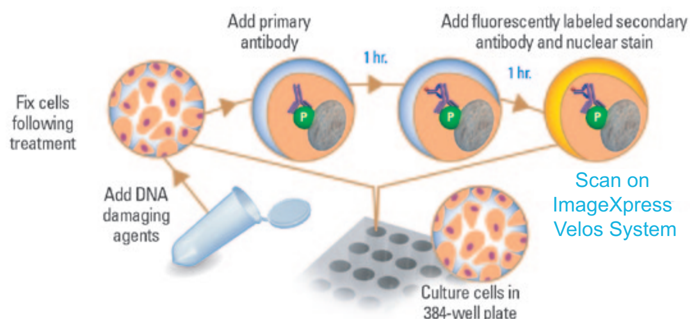

Figure 1. Cartoon of DNA damage assay performed by measurement of phosphorylated histone H2AX.

Assay Procedure

Cells and Culture Conditions. HeLa cells were cultured in DMEM supplemented with 10% FCS. Cells were plated at 384-well, black walled, clear-bottomed polystyrene plates (Greiner) at a density of 600 cells/well.

Detection of DNA Damage by ImageXpress Velos System. After RNAi and/or DNA damaging agent treatment, the cells were fixed, permeabilized and immunostained with an antibody directed against the phosphorylation of Ser139 of histone H2AX (Cell Signaling). An anti-rabbit Alexa Fluor-488 (AF488, Invitrogen) labeled secondary antibody detected the primary antibody and propidium iodide (PI) was used to identify all nuclei. The **ImageXpress Velos** laser scanning platform was setup for 2-channel acquisition with green (Ch1, 510-540nm BP) and red (Ch3, 600nm LP) filter channels. The image acquisition was done at 10 x 10 micron sampling. Whole well images were analyzed on a cell-by-cell basis by identifying cells in the red channel and using the background corrected integrated fluorescence intensity for the green and red channels. Representative images of positive and negative control wells are shown in **Figure 2.**

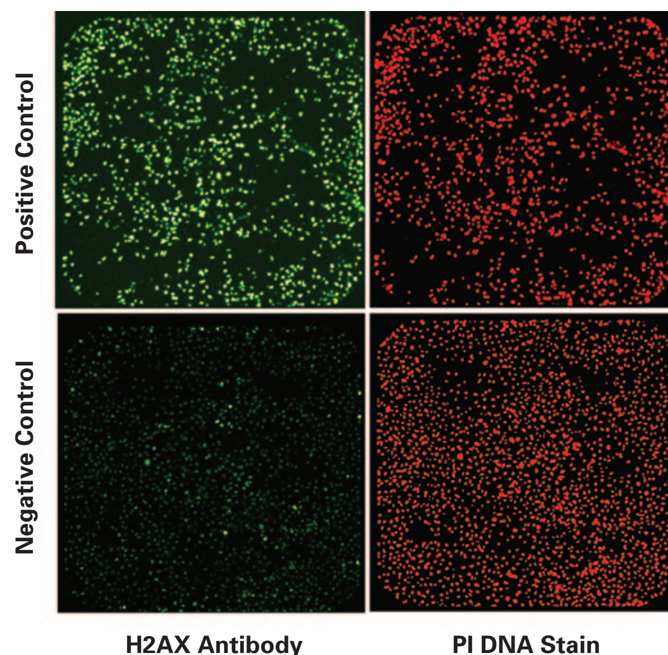

Figure 2. ImageXpress Velos System whole well images of positive and negative control wells from DNA damage assay. Images are of 384 well plate from the green (left) and red (right) channels.

Results & Discussion

Automated High Throughput DNA Damage Assay

The **ImageXpress Velos System**-HTS integrated with a Twister II Microplate Handler (Caliper) was used for the screening of a >20,000 RNAi library (in collaboration with Karlene Cimprich laboratory, Stanford University). The platform enabled walk-away auto-

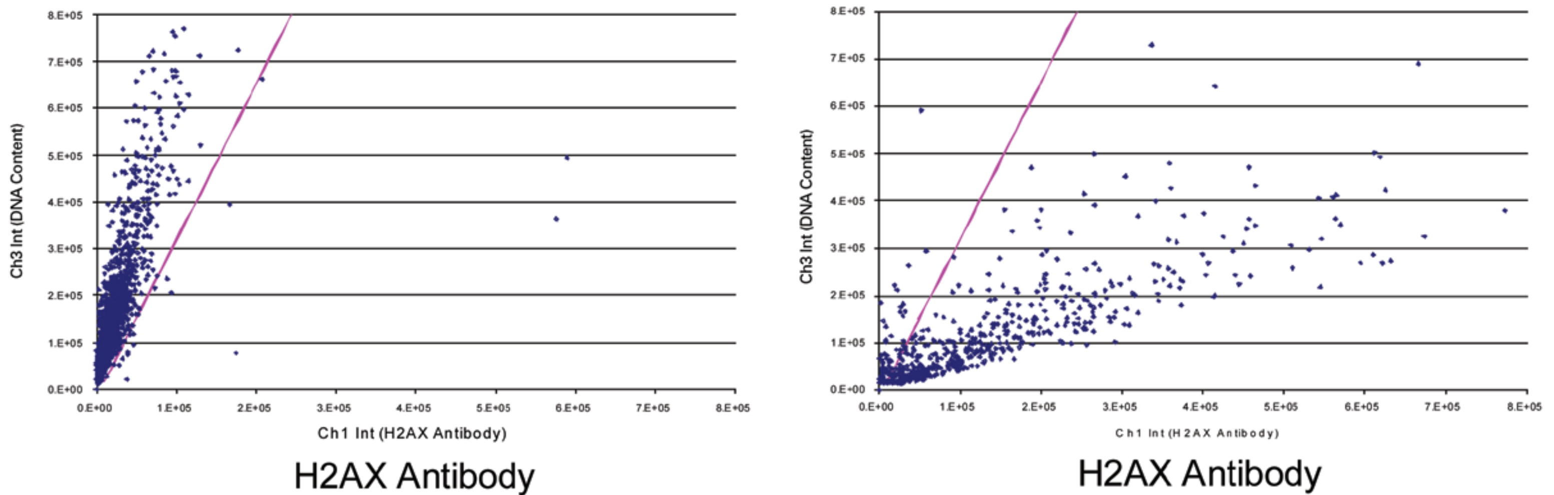

Figure 3. Cell-by-cell scatter plots of data from negative (left) and positive (right) control well images shown in **Figure 2**. The magenta line shows the Ch3/Ch1 criteria used for automatic DNA damage quantitation. The negative control was 1.9% and the positive control was 88.5%.

03B	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1.5	1.8	2.1	1.8	1.4	5.8	5.7	5.7	2.9	1.1	1.6	9.5	1.0	2.7	1.2	3.0	5.9	3.2	8.0	9.8	1.6	3.7	5.7	4.8
B	0.9	1.1	0.8	1.3	5.0	1.2	3.1	1.1	2.6	1.1	3.0	1.1	3.8	2.4	15.0	2.6	1.4	11.3	0.2	2.6	0.9	3.1	4.0	8.0
C	2.0	1.7	81.3	82.6	1.9	6.3	2.0	4.1	2.6	0.8	1.3	2.4	4.9	1.1	5.3	5.7	4.1	4.6	1.3	10.1	0.8	1.6	4.9	3.0
D	1.6	1.1	86.9	85.9	1.4	2.4	2.7	10.5	7.7	0.7	2.5	3.4	2.2	1.6	0.4	4.4	1.9	1.4	3.6	7.3	2.8	1.1	4.7	2.2
E	2.3	1.3	2.4	2.4	2.5	3.3	1.9	1.5	1.9	3.2	1.9	1.4	1.1	1.7	1.2	0.5	14.7	0.8	1.2	3.2	1.5	7.6	2.2	3.9
F	1.4	1.3	2.1	3.1	2.0	0.5	1.9	0.7	3.2	0.6	3.1	0.7	3.8	0.3	2.7	0.7	0.2	0.7	0.5	0.3	0.9	0.7	1.2	2.2
G	2.4	1.3	1.1	1.5	6.6	1.3	6.7	1.6	1.5	5.3	2.3	11.9	5.0	5.0	4.3	1.5	1.2	1.7	0.9	2.8	0.8	3.9	1.9	4.0
H	1.4	1.0	0.7	0.9	0.7	0.8	0.9	1.1	0.4	1.0	0.7	0.6	0.3	0.3	1.0	0.4	0.7	0.8	0.7	0.7	0.7	0.8	2.0	2.0
I	2.1	1.6	1.5	1.4	0.8	5.8	2.0	4.4	4.5	1.2	1.7	0.9	2.5	1.2	5.3	4.5	7.9	3.8	6.3	3.5	3.0	2.9	1.1	2.7
J	1.0	1.4	1.1	0.6	1.0	0.6	0.6	1.1	1.0	1.0	0.5	0.6	0.5	0.7	0.4	0.9	0.7	1.1	0.5	0.6	1.0	0.8	0.9	1.8
K	2.1	1.6	86.9	86.7	1.4	2.1	1.7	1.6	2.3	2.0	0.8	1.8	0.0	0.9	0.2	0.7	7.8	2.0	1.6	2.4	1.6	3.3	3.8	3.9
L	1.4	0.9	84.8	81.8	1.5	0.8	1.0	1.4	1.0	1.0	0.8	0.9	1.2	1.8	0.9	0.6	1.0	0.3	1.1	1.4	1.5	1.0	2.5	1.8
M	1.8	2.2	3.3	2.8	1.5	1.0	1.0	9.1	7.6	2.7	1.8	2.3	4.5	6.9	1.2	1.6	1.1	0.8	13.5	1.2	3.4	0.8	2.8	9.5
N	1.2	1.5	2.4	2.4	0.7	0.8	1.6	0.6	1.1	0.9	0.5	0.4	0.5	0.7	1.1	0.8	1.1	1.1	1.0	0.9	0.5	1.2	1.1	1.9
O	2.1	1.4	1.3	2.2	0.4	4.7	6.9	2.4	7.5	7.6	4.4	8.4	1.9	2.3	26.1	2.8	2.3	26.0	2.1	2.3	2.7	2.9	16.8	8.1
P	0.8	1.0	1.1	1.3	0.7	1.2	1.1	0.8	1.9	1.3	1.3	1.1	1.0	1.1	0.9	1.2	1.0	1.0	1.2	1.9	5.3	2.1	1.8	1.7

Figure 4. Representative plate layout and percent DNA damage per well results of RNAi screen in 384-well plate format. The negative control wells (n=8) are shown in gray and the positive control wells (n=8) are shown in green. The typical Z'-prime value was 0.9. All other wells were treated with RNAi and wells with values ≥13.5% are shown in orange.

mated image acquisition and automatic cell quantitation of DNA damage by a two-color image processing process. Two-parameter dot plot results for phosphorylated H2AX and PI staining of cells for the negative and positive control treated samples from **Figure 2** are shown below in **Figure 3**. The dot plots shown were generated using an Excel macro developed by BBI. The purple dividing line represents the Ch3/Ch1 ratio with an adjustable x-axis intercept that defines the settings used for automatic DNA damage quantitation. The negative control sample was 1.9% and the positive control (RNAi previously shown to increase DNA damage) was 88.5%.

The percent DNA damage results from a representative 384-well plate are shown below in **Figure 4**. The negative control wells (n=8) are shown in gray and the positive control wells (n=8) are shown in green. The typical Z'-prime value was 0.9. All other wells were treated with RNAi and wells with values ≥13.5% are shown in orange.

Conclusions

The phosphorylation of histone H2AX has been shown to be a sensitive indicator of DNA double-strand breaks produced by drugs or ionizing radiation. In this report we have shown how the **ImageXpress Velos**-HTS laser scanning platform has enabled a high throughput 2-color assay for DNA damage quantitation. We also have demonstrated that this two-color DNA damage assay is a sensitive and fast automated process for RNAi screens designed to identify genes that sensitized cancer cells to DNA damaging agents (manuscript in preparation). The unique optics and scanning engine of this platform enables simple "plug and play" applications to meet the needs of life science researchers in both academia and industry.

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