

# Quantitative Analysis of Genotoxicity and Cytotoxicity to DNA Damaging Agents Using High-Content Imaging

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## Abstract

A double-strand break (DSB) in genomic DNA is potentially a lethal lesion. One of the known responses to DSB formation is phosphorylation of H2A histones. Specifically, exogenous or endogenous agents that lead to DNA damage induce phosphorylation of histone variant H2AX at Ser139 forming DNA foci at the site of DNA DSBs. Phosphorylated H2AX aids in the recruitment of proteins responsible for double-strand break repair. In mammalian cells, phosphatidylinositol 3-kinase-like protein kinases such as ATM (ataxia-telangiectasia mutated), ATR (ATM- and Rad3-related), and DNA-PKcs (DNA-dependent protein kinase catalytic subunit) phosphorylate histone variant H2AX. In general, cells have intrinsic mechanisms to adapt to very low levels of irreparable damage. However, if a DSB inactivates an essential gene or triggers apoptosis, it can be sufficient to kill a cell. DNA DSBs are biologically very important because their repair is intrinsically more difficult than that of other types of DNA damage. Thus, the intrinsic ability of cells to monitor DSB formation is essential to the viability of the cell.

We developed an assay to measure the effects of compounds or drugs that induce DSBs in cells. This assay enables the simultaneous quantitation of two cell health parameters in the same cell by high content analysis: genotoxicity and cytotoxicity. DNA damage is measured as an indicator of genotoxicity using specific antibody-based detection of phosphorylated H2AX (Ser139) in the nucleus. Cytotoxicity is measured using a new dead cell dye that does not stain nuclear DNA in intact live cells due to the impermeability of the plasma membrane for this probe. Drugs and test compounds leading to serious cell injuries, including plasma membrane permeability, allow entry of the dye and reflect general cytotoxicity. A549 and HeLa cells treated with a variety of compounds known to induce DNA damage were used to validate the consistency and robustness of this assay for cytotoxicity measurements. The data demonstrated that this multi-parametric approach revealed cells which underwent pre-lethal DNA damage while lethal damage was induced in other cells within the same wells, underscoring the value of high content imaging-based assays in cytotoxicity. In addition to pH2AX detection and determination of plasma membrane integrity, compatibility with a new click chemistry-based Tdt-mediated dUTP nick end labeling (TUNEL) assay for DNA damage is shown.

## Figure 1. DNA Damage Response Pathway

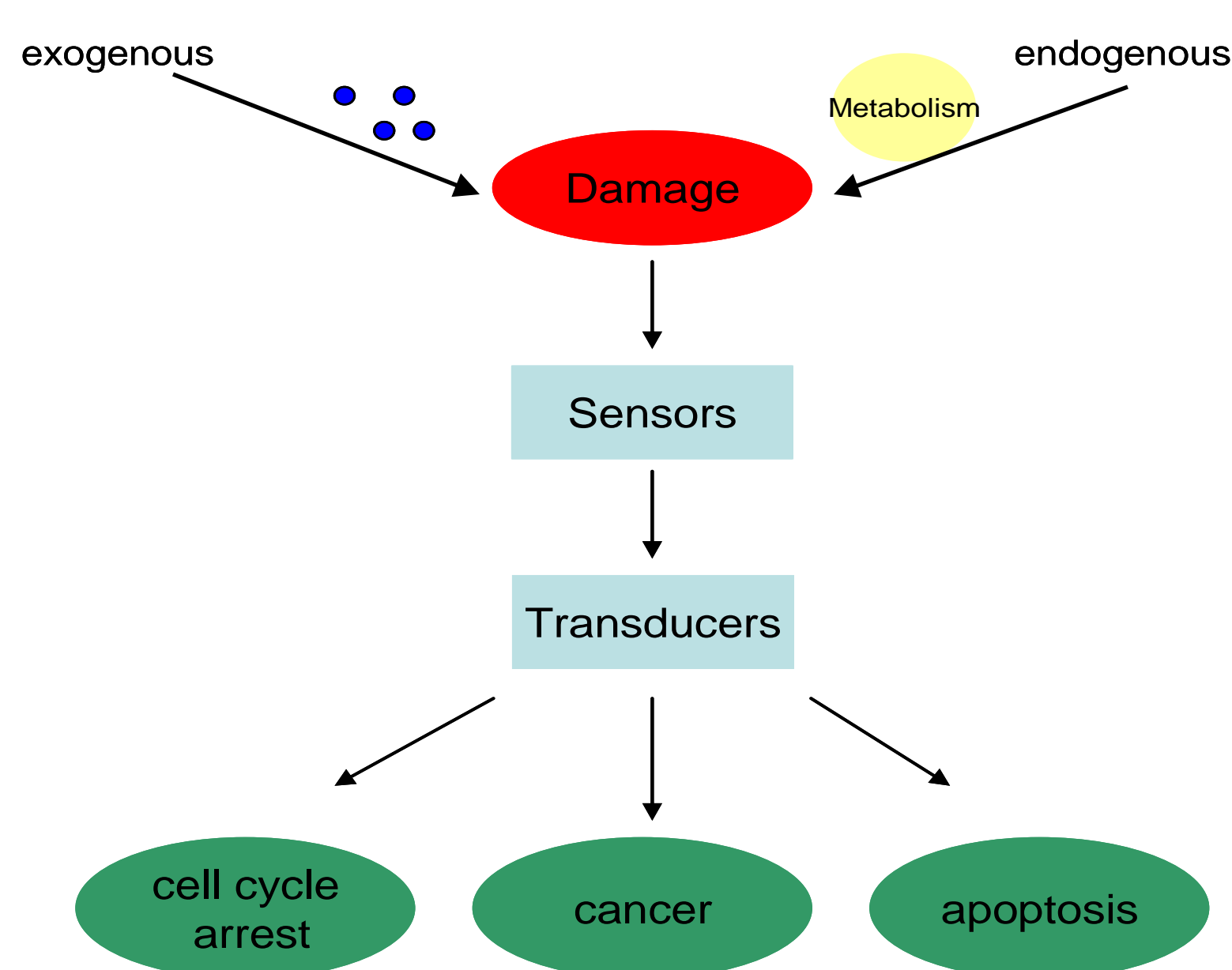
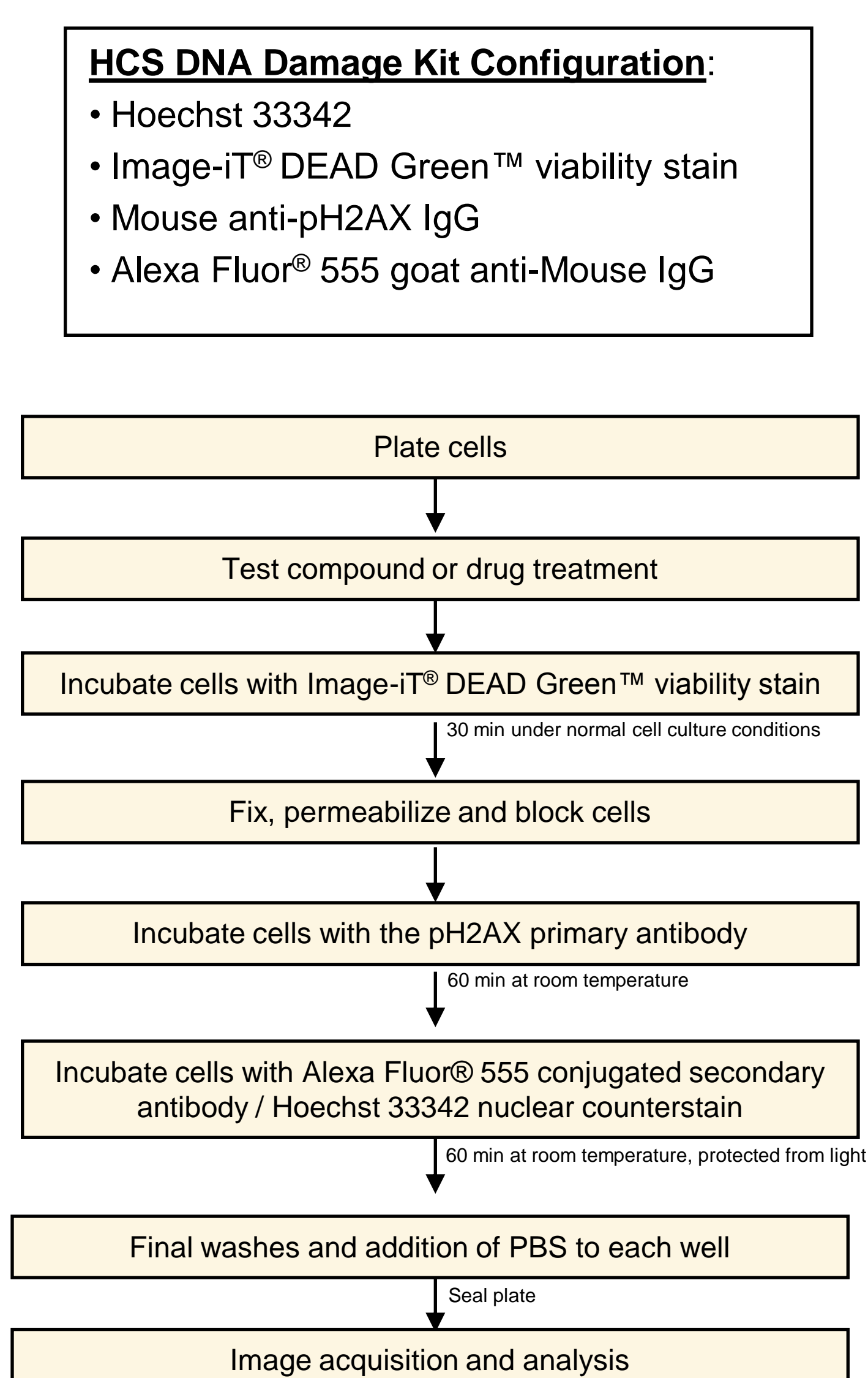


Figure 1. DNA damaging agents induce DSBs that could lead to reversible or irreversible damage in cells. Irreparable damage could lead to genomic instability, tumorigenesis or cell death.

## Figure 2. HCS DNA Damage Assay Work Flow



### HCS DNA Damage Kit Configuration:

- Hoechst 33342
- Image-iT® DEAD Green™ viability stain
- Mouse anti-pH2AX IgG
- Alexa Fluor® 555 goat anti-Mouse IgG

## Figure 3. Multiplex Imaging of Genotoxicity & Cytotoxicity

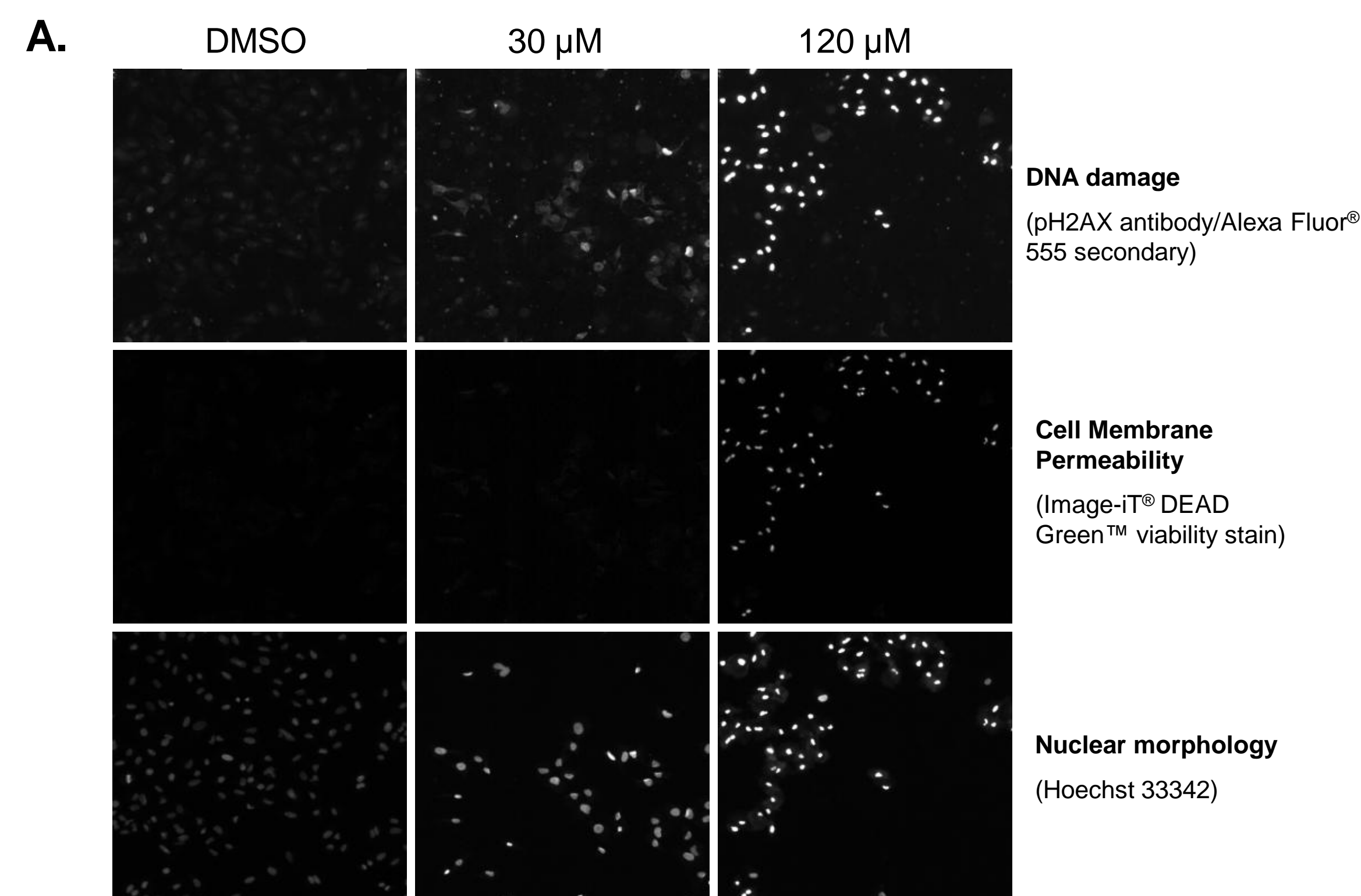


Figure 3. A549 cells were treated with increasing concentrations of valinomycin for 24 hours and showed genotoxic and cytotoxic effects as indicated by pH2AX antibody labeling and the Image-iT® DEAD Green™ viability stain. 30 μM valinomycin treatment resulted in an pre-lethal increase of pH2AX but not loss of plasma membrane integrity.

## Figure 4. Imaging of Genotoxicity & Cytotoxicity

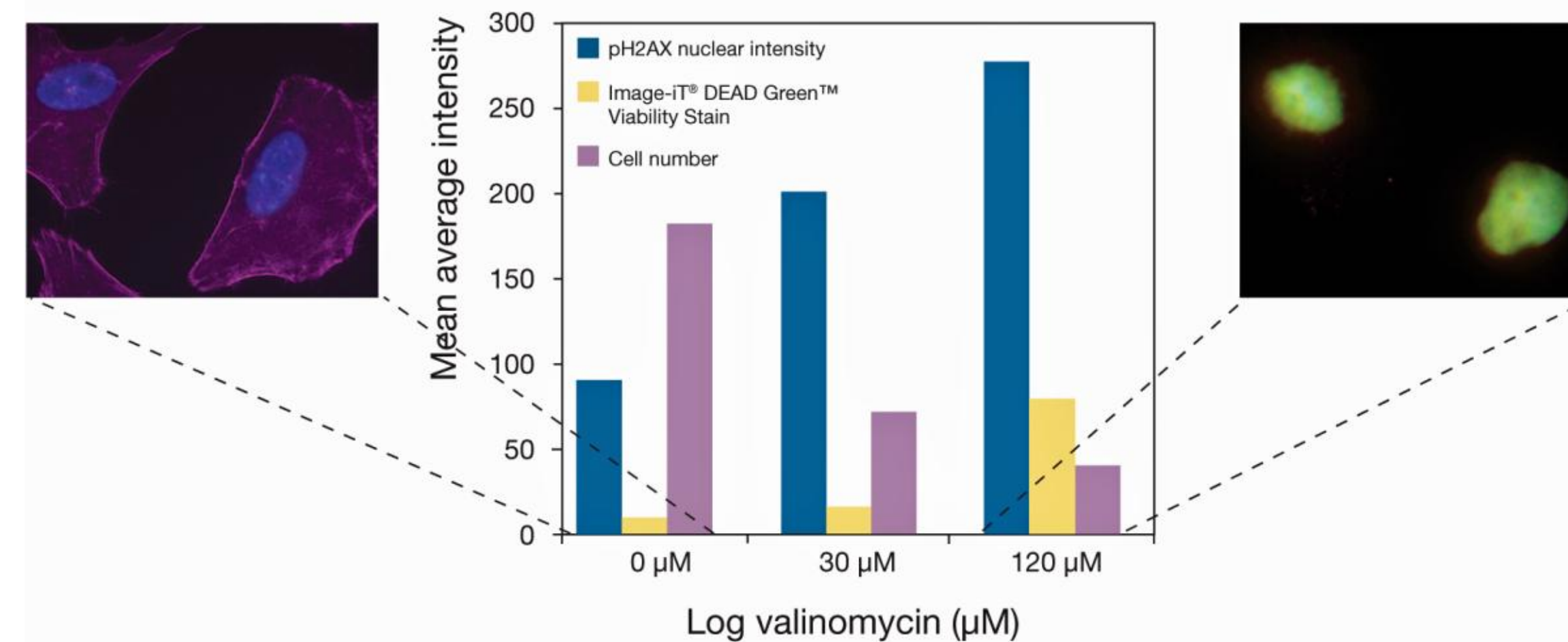


Figure 4. Quantitative representation of DNA damage, cytotoxicity, and cell loss with increasing concentrations of valinomycin in A549 cells. The DNA damage assay with F-actin and nuclear counterstaining (Alexa Fluor® 647 phalloidin – purple, Hoechst - blue) was performed on fixed and permeabilized cells. The left-side image represents untreated cells with intact F-actin cytoskeleton and no evidence of toxicity. The right-side image illustrates both genotoxicity and cytotoxicity (pH2AX detected with Alexa Fluor® 555 secondary – orange and Image-iT® DEAD Green™ viability stain - green) as well as completely disrupted actin cytoskeletons in cells treated with 120 μM valinomycin.

## Figure 5. Dose Response - Valinomycin, Etoposide, H<sub>2</sub>O<sub>2</sub>

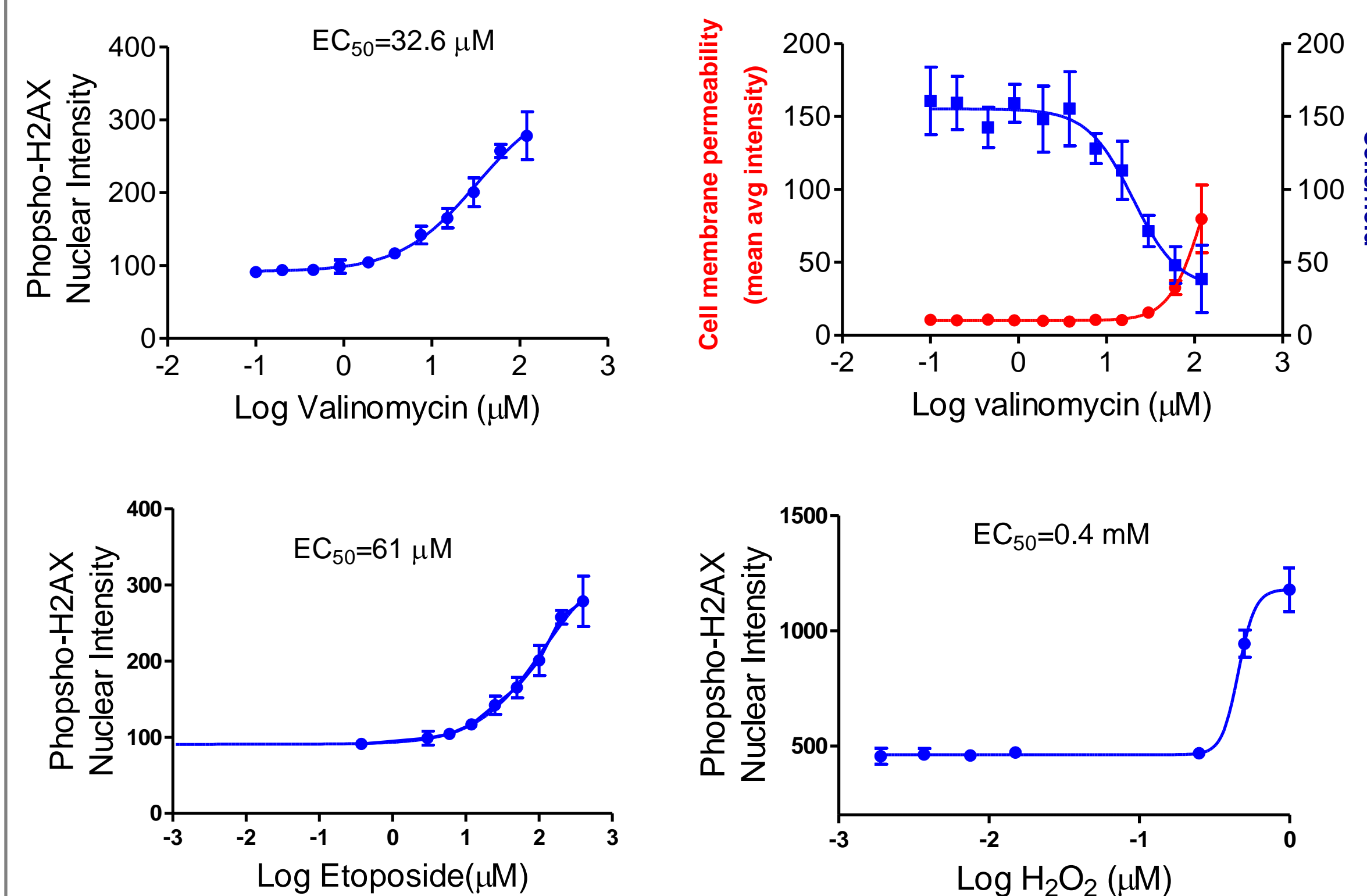


Figure 5. A549 cells were treated with valinomycin, etoposide and H<sub>2</sub>O<sub>2</sub> for 24 hours and assayed using the HCS DNA Damage Kit. EC<sub>50</sub> values were generated for the three test compounds.

## Figure 6. HCS DNA Damage Assay: Robustness & Consistency

Measured Parameter	%CV	Z factor	Fold Change
DNA Damage (valinomycin)	13.5 3 3.6	0.47 0.05	10.2 1.1
Image-iT® Dead Green™ Viability Stain	15.3 3.5	0.37 0.16	12.28 1.4

Figure 6. The data represents robustness and consistency achieved in measuring DNA damage and cytotoxicity between 3 min/max plates.

## Figure 7. Induction of Genotoxicity by DNA Damaging Agents

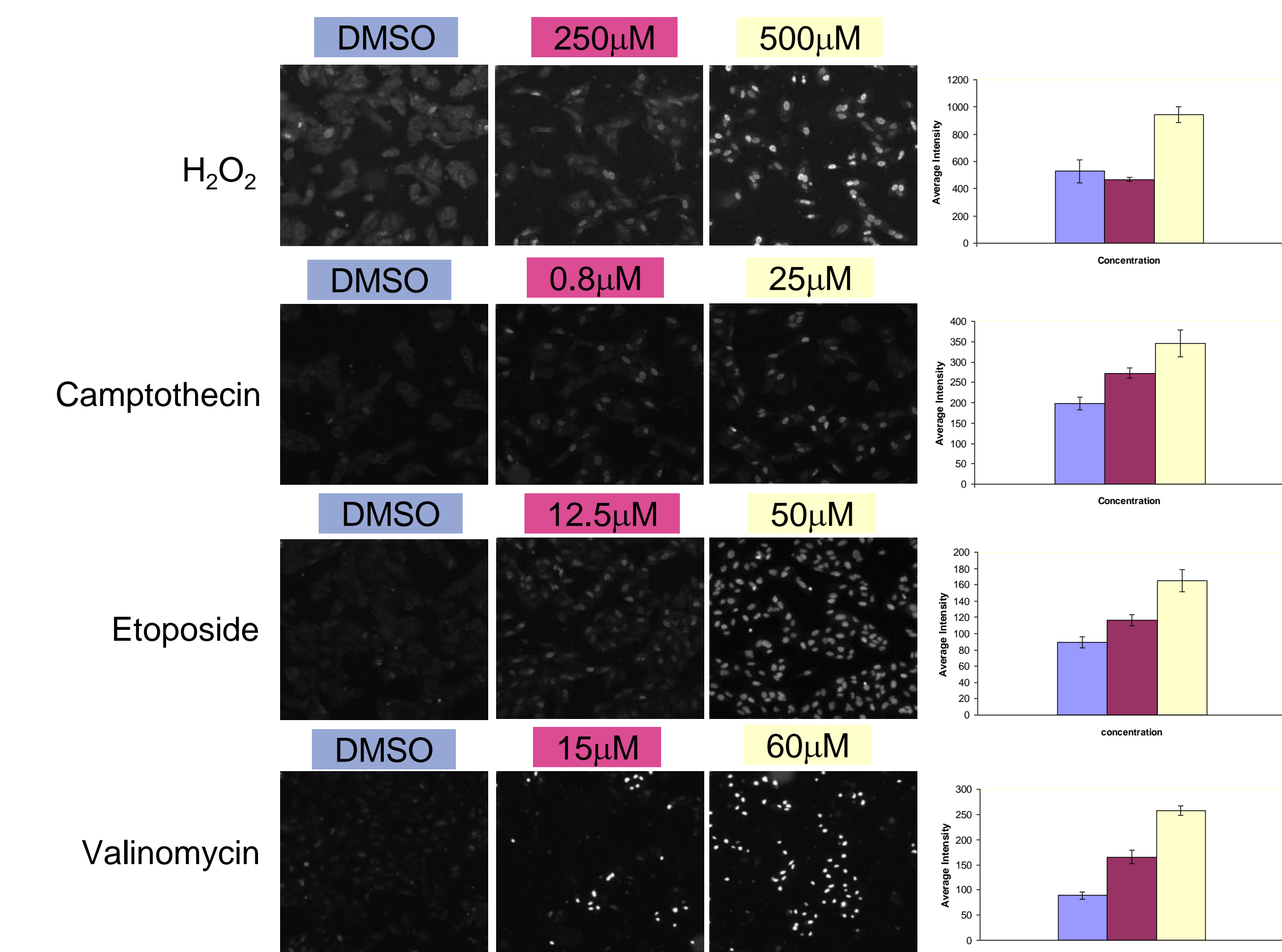


Figure 7. A549 cells were treated with increasing concentrations of H<sub>2</sub>O<sub>2</sub>, camptothecin, etoposide, and valinomycin before fixation and permeabilization. Detection of pH2AX using the HCS DNA Damage Kit revealed increased levels of pH2AX in the nuclear regions of treated cells which was readily quantifiable.

## Figure 8. Multiplex Assessment of DNA Damage: Combining the Click-iT® TUNEL Assay with pH2AX Detection

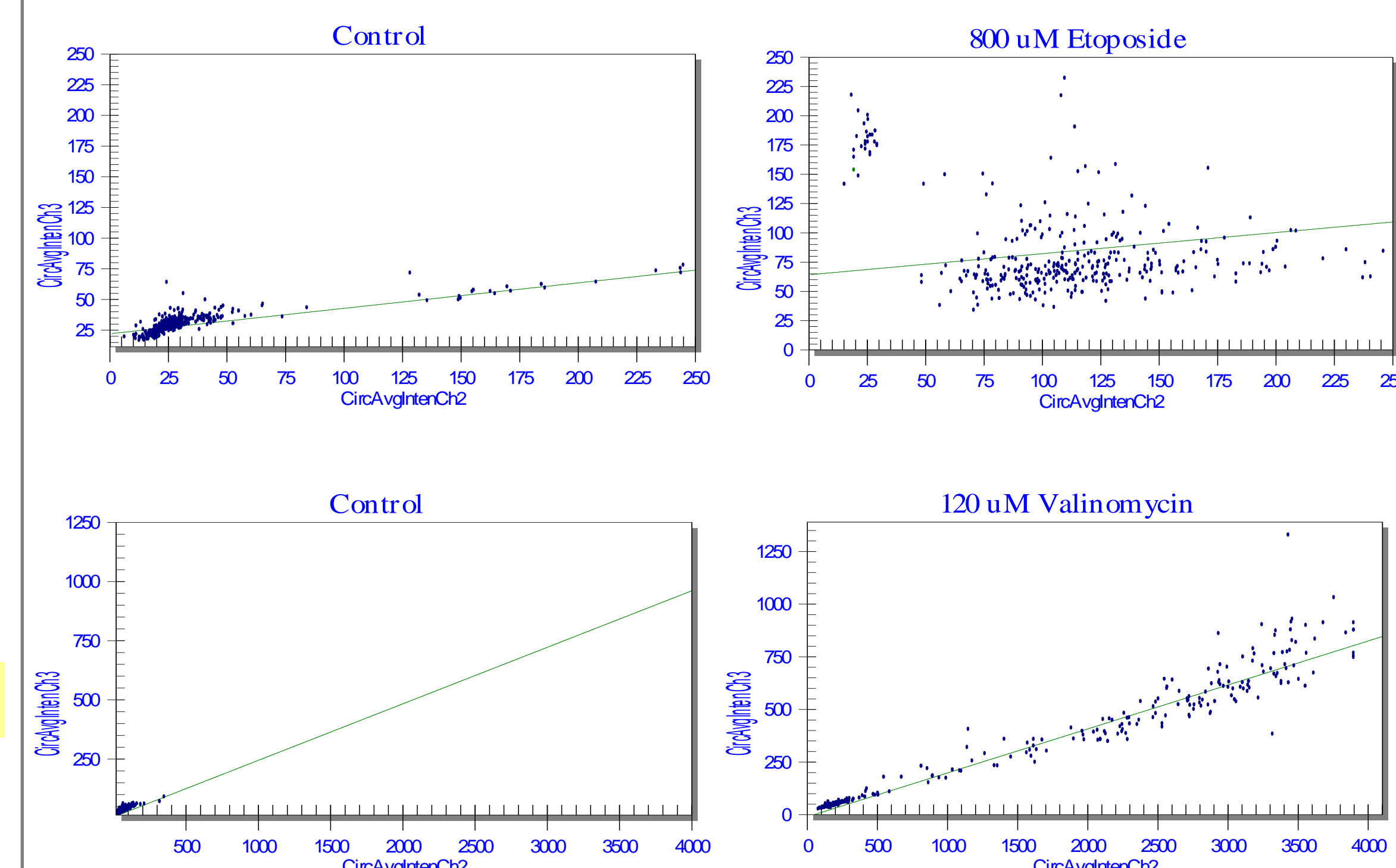


Figure 8. A549 cells were treated with etoposide or valinomycin followed by assessment of DNA damage using the click chemistry-based Tdt-mediated dUTP nick end labeling (TUNEL) assay and the HCS DNA Damage Kit. Most, but not all cells treated with 800 μM etoposide or 120 μM valinomycin showed significant increases in both TUNEL (Ch2) and pH2AX (Ch3), demonstrating that these two assays may be combined to provide mechanistic insight in drug profiling with respect to genotoxicity.

## Conclusions

- The HCS DNA Damage Kit (H10292) enables sensitive and simultaneous detection of genotoxicity (pH2AX) and cytotoxicity (plasma membrane integrity) by automated imaging and analysis.
- The multi-parametric nature of the HCS DNA damage assay revealed drug concentrations at which only pre-lethal genotoxicity was observed while higher drug concentrations induced both genotoxicity and a lethal loss of plasma membrane integrity.
- The DNA damage assay is robust and consistent as indicated by a coefficient of variation that is less than 20%, Z factors >0.3, and by valinomycin-induced increases in pH2AX and Image-iT® DEAD Green™ (110291) signals of >3-fold and >10-fold, respectively.
- The HCS DNA damage assay can be combined with other assays for DNA damage such as the Click-iT® TUNEL assay (A10028, C10081, C10082), a biomarker typically associated with apoptosis, to facilitate more mechanistic insight in drug profiling with respect to genotoxicity.

## References

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