

Assessment of Ultraviolet Light- and Chemical-Induced UDS in Various Cell Lines Using Flow Cytometry

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ABSTRACT

UDS is a reliable assay to measure DNA damage/repair induced by genotoxins and ultraviolet (UV) radiation. Standard UDS assays detect radiolabeled nucleotides incorporated into repaired DNA. We have developed a FLOW cytometry-based UDS assay (FLUDS) that measures biotin-dUTP incorporation into repaired DNA. Cultured cells were exposed to DNA damaging agents, including direct, metabolically activated genotoxins and ultraviolet light (UVB and UVC). Aphidicolin (Aph), a DNA repair enzyme inhibitor, was included in cell cultures exposed to DNA-damaging agents to temporarily inhibit repair and allow DNA damage to accumulate prior to the UDS step. The addition of Aph helps enhance the signal-to-noise (S/N) ratio.

Using a human skin keratinocyte cell line, FLUDS exhibited 90% accuracy, 100% sensitivity, and 85% specificity in detecting direct-acting genotoxins, correctly categorizing 8 of 10 direct-acting genotoxins and 11 of 11 non-genotoxins. All positive responses were in a clear dose-dependent manner. In the case of Methyl methanesulfonate (MMS), a distinct population of biotin-dUTP positive cells increased from 1.8% to 61% of gated G0/1 cells in response to increasing concentrations from 10 to 100 μ M. Less than 1% UDS+ cells were detected in vehicle control cultures as background. When testing bioactivation-dependent genotoxins, FLUDS detected 4 of 19 procarcinogens, which may be attributed to metabolic activation occurring in skin cell types.

We successfully detected UDS by FLUDS assay in various other cell lines, HEK293, HepG2, PC3, A431, L929, 3T3, and MDA-MB-157. However, the signal to noise ratios were lower than that in human keratinocytes. In summary, the validated FLUDS assay is quantitative in nature, eliminates the need for the use of radioactive materials, is amenable to use in various cell lines, and thus obviates the need for use of laboratory animals.

INTRODUCTION

The *in vitro* Unscheduled DNA Synthesis (UDS) assay is extremely useful for identifying and characterizing genotoxic agents that potentially induce damage in genomic DNA. However, the application of UDS was limited by the radioactive material used, the extremely long autoradiographic procedures, and subjective examination. To overcome those drawbacks, we have developed an *in vitro* UDS detection system using flow cytometry technology (FLUDS). Biotin-dUTP, a thymidine analog, is incorporated into newly synthesized DNA strands using the cell's own repair mechanism (UDS) induced by genotoxins. Flow cytometry is applied to detect the FITC-conjugated monoclonal anti-biotin antibody bound to the biotin-dUTP incorporated in the repaired DNA. Meanwhile, DNA staining allows the analysis of DNA repair in the specific cell cycle phases.

The original FLUDS suffered from poorly reproducible and inconsistent results due to the low signal-to-noise ratio. Aphidicolin, a reversible DNA repair enzyme inhibitor, was introduced either in the chemical treatment step or after ultraviolet radiation for inhibition of rapid repairing mechanism prior to the addition of biotin-dUTP, in order to increase the signal. Addition of aphidicolin dramatically enhanced the sensitivity of FLUDS, especially when HaCaT cells, an immortalized human keratinocyte cell line, were used in the systems. Keratinocytes are the major constituent of skin, which is the largest organ, and is topically and frequently exposed to potential environmental genotoxins, such as UV radiation. HaCaT cells could therefore serve as a useful human skin model in a UDS study.

The validation of FLUDS was performed in HaCaT cells with 10 direct-acting genotoxins and 11 non-genotoxins, as well as 19 known metabolic-activation-required genotoxins. UVA/B irradiation was reported to cause alternative DNA synthesis in skin. It is interesting to evaluate the effect of UVA/B on UDS in HaCaT cells by FLUDS assay.

Other cell lines besides HaCaT cells used in FLUDS would give the assay broader applications in genotoxicity studies, such as look for specific organ target for a particular genotoxin.

METHODS AND MATERIALS

Cell Culture: HEKa (Invitrogen), A431 (ATCC), L929 (ATCC), 3T3 (ECACC), HepG2 (ATCC), PC3 (ATCC), HaCaT (gift), and MDA-MB-157 (ATCC) cells were cultured using appropriate culture medium (as suggested by supplier) on 24-well plates. After the cells were confluent, they were starved with unsupplemented medium for three days, except for primary cells, MDA-MB-157 and HepG2, which were only starved for one day, to increase the population of cells in G0/G1 phase.

Dosing: After starving, cells were treated with unsupplemented media with 30 μ M aphidicolin (Aph) and chemical treatment for 4 or 18 hours followed by harvest using trypsin. For UV dosing, media was aspirated, HBSS with 30 μ M Aph was added, and cells were treated with either UVA- 0-23J/cm² (Honle solar simulator "500 S", H1 filter), UVB- 0-0.20 J/cm² (Honle solar simulator "500 S", H2 filter) or UVC- 0-20 J/m² (Philips TUV 30W T8, germicidal UVC) radiation. All detectors (PMA2110 for UVA, PMA2106 for UVB, and PMA2122 for UVC) and radiometers (PMA2200) used were supplied by Solar Light Co., Inc. After UV dosing, unsupplemented media with 30 μ M Aph was added. Following a 4-hour incubation, cells were harvested using trypsin.

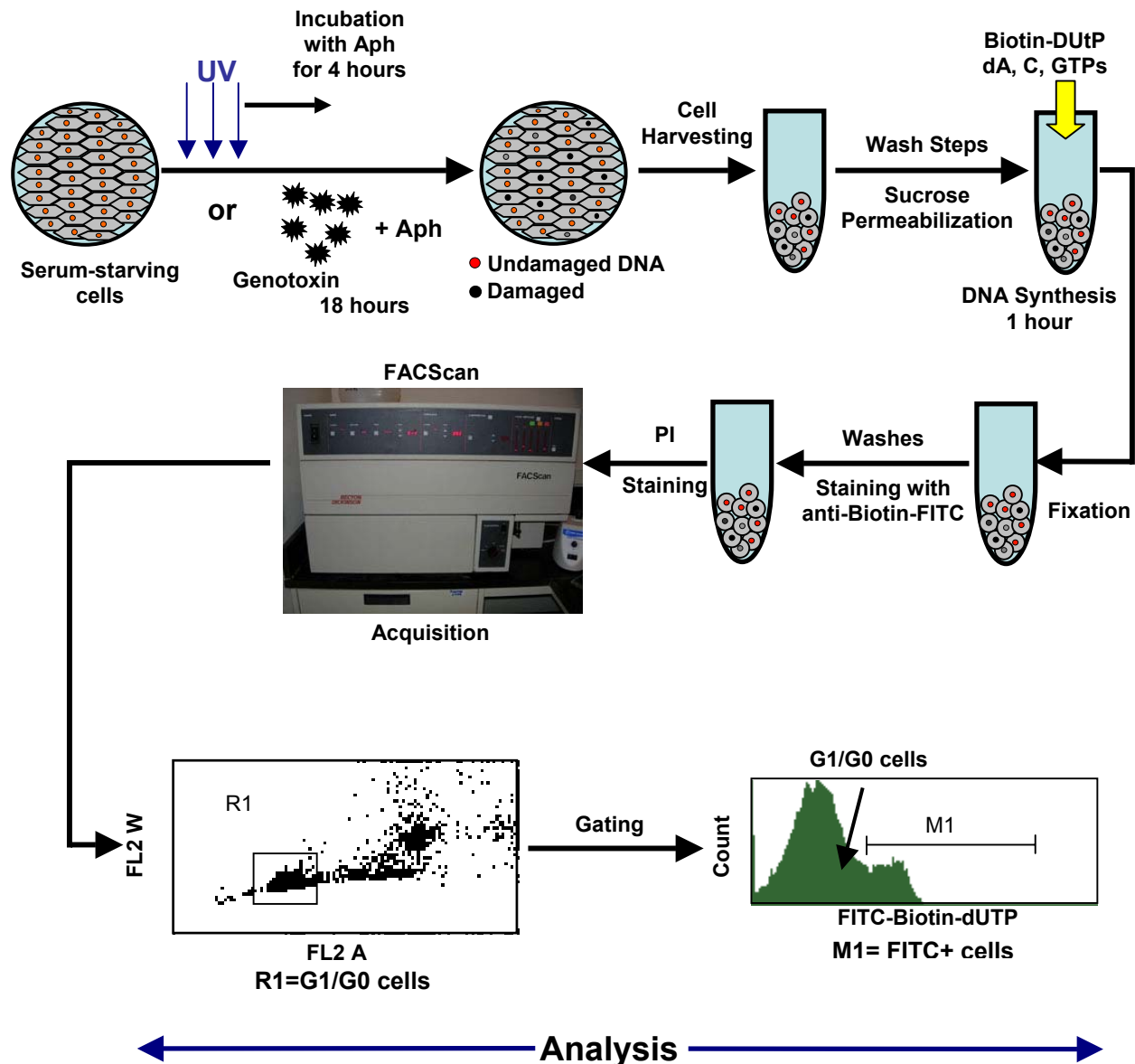
Permeabilization and Synthesis: Following harvest the cells were centrifuged, washed, and the cell pellets were re-suspended in a permeabilization buffer (50 mM Tris, 10 mM MgCl₂, 1 mM DTT, 250 mM sucrose and 1 mM EDTA, pH 8.0). Following a 20-minute incubation on ice, the cells were again washed and centrifuged. The cell pellets were re-suspended in a synthesis mix (50 mM Tris, 10 mM MgCl₂, 1 mM DTT, 125 mM sucrose, and 2.5 mM ATP, pH 8.0). DNA repair synthesis was initiated by adding all necessary dNTPs (dATP, dGTP, and dCTP, 100 μ M each) (Clontech, Mountain View, CA) to cells, along

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with the biotinylated thymidine analog, Biotin-dUTP (Clontech). Reactions took place in a humidified CO₂ incubator at 37°C for 1 hour. After being washed and spun down, the processed cells were fixed with cold 90% methanol and incubated on ice for at least 30 minutes.

Staining: The fixed cells were intensively washed and re-suspended in a staining buffer (0.5% Tween-20, 1% BSA, 0.03% sodium azide in PBS) containing a FITC-conjugated anti-biotin antibody. Following a 45-minute incubation on ice, the cells were washed and stained with propidium iodide DNA labeling. The labeled cells were analyzed by flow cytometry using a FACScan (BD, San Jose, CA). In the DNA staining profile, cells in G1 phase were gated to avoid cell debris, cell aggregates, and cells in S and G2/M phase. The marker was appropriately set for FITC+ (Biotin-dUTP+ or UDS+) cells by using a vehicle or negative control as a reference.

PROCESSING



RESULTS:

FLUDES VALIDATION ON HaCaT CELLS

Chemical (Sigma)	CAS#	Doses Evaluated	Overall Result	Maximal Responses	Lowest Effective Response
Direct-acting Genotoxins					
Mitomycin C	50-07-7	0.1-20 µM	Neg*	10 µM	10 µM
Actinomycin D	50-76-0	130-4000 nM	Neg*	150 nM	1 µM
Daunorubicin hydrochloride	23541-50-6	30-10000 nM	POS	3000 nM	300 nM
Ethidium bromide	1239-45-8	625-50000 nM	POS	50000 nM	1250 nM
N-nitroso-N-methylurea	684-93-5	30-10000 µM	POS	10000 µM	3000 µM
N-nitroso-N-ethylurea	759-73-9	40-10000 µM	POS	10000 µM	5000 µM
4-Nitroquinoline N-oxide	56-57-5	1.56-12.5 µM	POS	12.5 µM	1.56 µM
Ethyl methanesulfonate	62-50-0	1.6-10000 µM	POS	5000 µM	1000 µM
Methyl methanesulfonate	66-27-3	0.32-250 µM	POS	100 µM	30 µM
Ultraviolet C	NA	0.2-20 J/m ²	POS	20 J/m ²	0.2 J/m ²
Metabolic-activation-required Genotoxins					
N-(2-Fluorenyl)acetamide	53-96-3	1.6-500 µM	Neg		
2-Aminoanthracene	613-13-8	0.16-40 µM	POS	0.63 µM	0.16 µM
2-Aminofluorene	153-78-6	1.6-500 µM	Neg		
Aflatoxin B1	1162-65-8	0.16-50 µM	POS	50 µM	16 µM
Benzo[a]pyrene	50-32-8	30-100000 nM	POS	3000 nM	300 nM
2,4-Diaminotoluene	95-80-7	31.3-1000 µM	Neg		
1,1-Dichloroethane	75-34-3	625-20000 µM	Neg*	20000 µM	1250 µM
1,2-Dichloroethane	197-06-2	31.7-31700 µM	Neg*	31700 µM	31700 µM
N-Nitrosodiethylamine	55-18-5	3-100 mM	Neg		
1-Nitrosopyrrolidine	930-55-2	30-10000 µM	Neg		
Azaserine	115-02-6	12.5-400 µM	POS	400 µM	50 µM
Quinoline	91-22-5	30-1000 µM	Neg		
Urethane	51-79-6	30-1000 µM	Neg		
Nitrosomorpholine	59-89-2	10-3000 µM	Neg*	3000 µM	3000 µM
7,12-Dimethylbenz[a]anthracene	57-97-6	6-200 µM	Neg*	13 µM	13 µM
Benz[a]anthracene	56-55-3	1.6-500 µM	Neg*	5 µM	50 µM
Phenazopyridine hydrochloride	136-40-3	6.25-200 µM	Neg*	100 µM	50 µM
Isophorone	78-59-1	94-10000 µM	Neg		
2,4-Dimethylaniline	95-68-1	12.5-10000 µM	Neg		
Non-Genotoxins					
Distilled Water	7732-18-5	0.2-5.0%	Neg		
Corn Oil (ACH food Company)	8001-30-7	0.2-5.0%	Neg		
Dimethylsulfoxide	67-68-5	0.2-5.0%	Neg		
Ethanol	64-17-5	0.2-5.0%	Neg		
2,6-Diaminotoluene	823-40-5	300-1000 µM	Neg		
Caprolactam	105-60-2	300-10000 µM	Neg		
Phenobarbital	50-06-6	200-5000 µM	Neg		
Pyrene	129-00-0	20-500 µM	Neg		
Aniline	62-53-3	300-10000 µM	Neg		
Reserpine	50-55-5	20-500 µM	Neg		
11-Aminoundecanoic acid	2432-99-7	300-2500 µM	Neg		

POS = Positive UDS response (threshold of positive response is 200% UDS+ of vehicle control)

Neg = Negative UDS response

Neg* = Positive in one or some doses, but not in dose-related manner

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Direct-Acting Agents:

	Known +	Known -	
Tested +	8	0	8
Tested -	2	11	13
	10	11	21

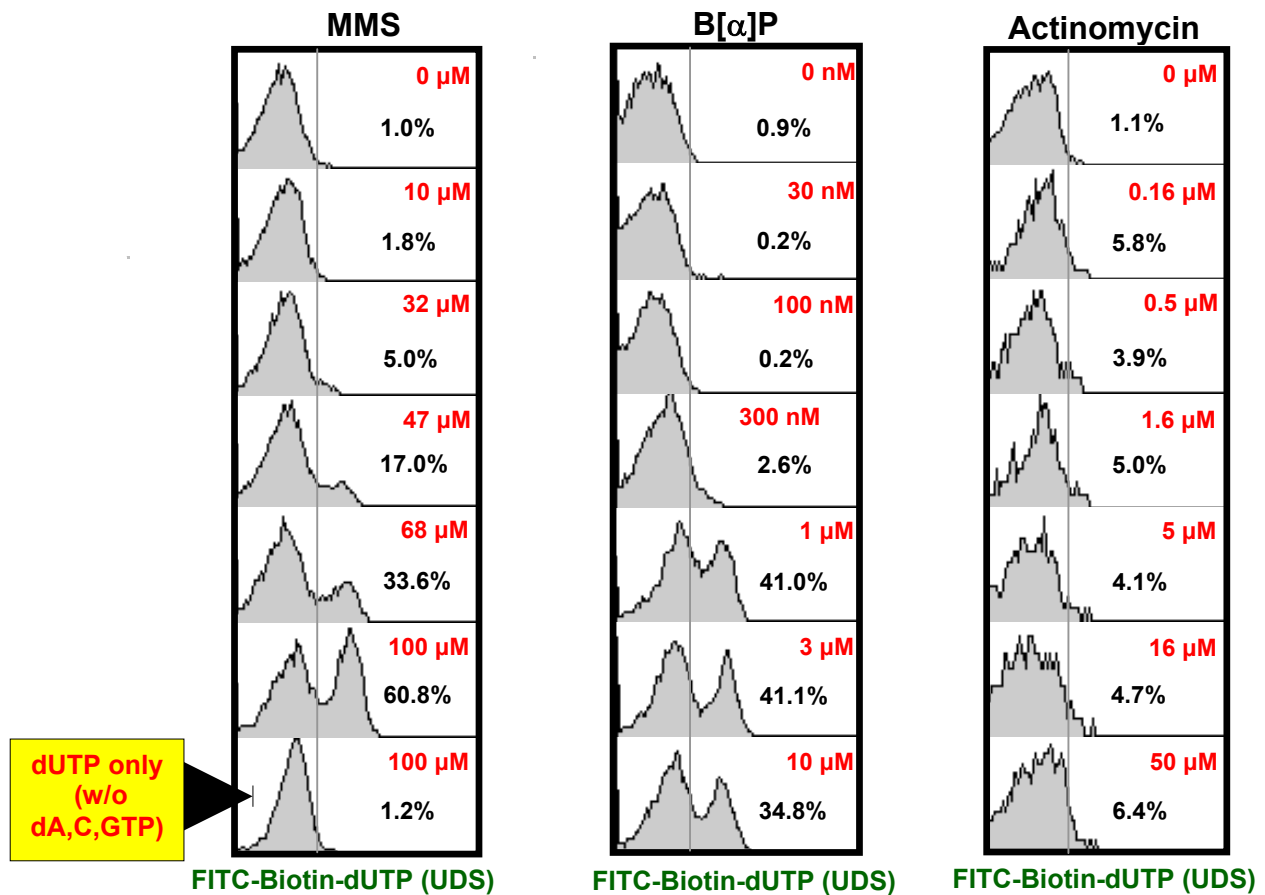
Accuracy	90%	(19/21)
Sensitivity	100%	(8/8)
Specificity	85%	(11/13)
Positive Predictivity	80%	(8/10)
Negative Predictivity	100%	(11/11)

All Listed Agents:

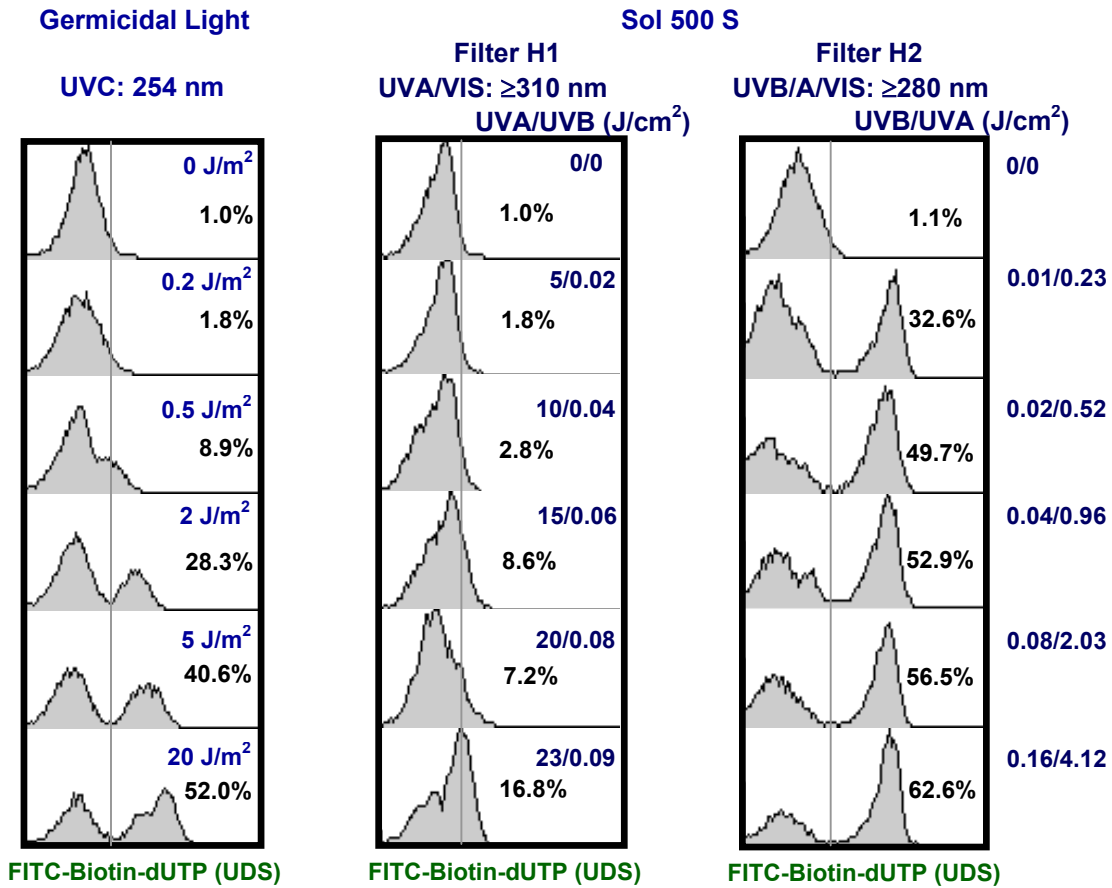
	Known +	Known -	
Tested +	12	0	12
Tested -	17	11	28
	29	11	40

Accuracy	58%	(23/40)
Sensitivity	100%	(12/12)
Specificity	39%	(11/28)
Positive Predictivity	41%	(12/29)
Negative Predictivity	100%	(11/11)

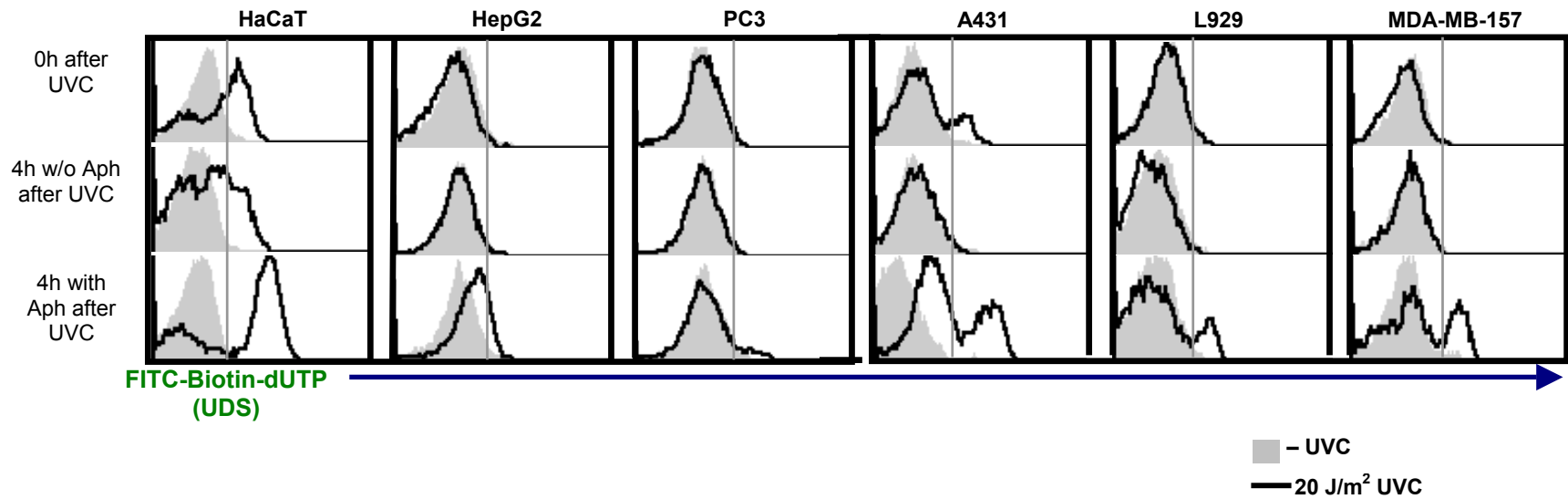
UDS INDUCED BY CHEMICALS



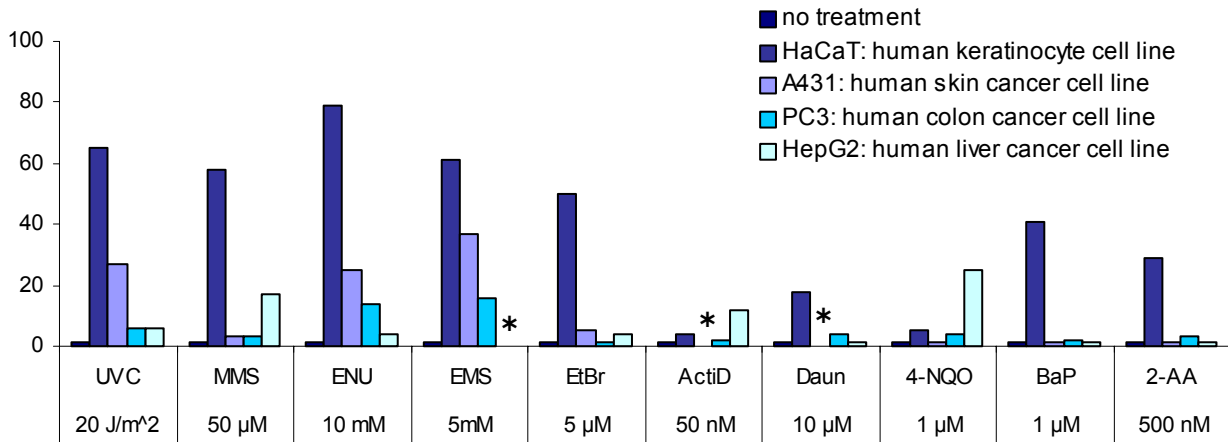
UDS INDUCED BY ULTRAVIOLET IRRADIATION



UDS INDUCED BY UVC (20 J/m²) IN DIFFERENT CELL LINES



Genotoxins Tested by FLUDS in Different Cell Lines



ENU: N-nitroso-N-ethylurea; **EMS:** Ethyl methanesulfonate; **EtBr:** Ethidium bromide; **ActiD:** Actinomycin D; **Daun:** Daunorubicin hydrochloride; **4-NQO:** 4-Nitroquinoline N-oxide; **BaP:** Benzo[a]pyrene; **2-AA:** 2-Aminoanthracene. * = Not tested

SUMMARY

A flow cytometry-based Unscheduled DNA Synthesis assay (FLUDS) had been developed and optimized in MB Research Laboratories to get high detection sensitivity, with 90% accuracy, 100% sensitivity, and 85% specificity in detecting direct-acting genotoxins by using HaCaT cells. We used the assay to detect the DNA repair synthesis induced by different ultraviolet irradiations in HaCaT cells, which may serve as a human skin model. The UV studies revealed that long time exposure with long wavelength of UV (UVA) also generated significant amount of UDS. Several other cell lines (HepG2, PC3, A431, L929, and MDA-MB-157) besides HaCaT were also successfully used in FLUDS to evaluate the UDS induced by chemicals and UVC, which could be potentially applied in FLUDS assay as other organ models in study of specific genotoxins.

CONCLUSIONS

- Validation of FLUDS in a human keratinocyte cell line, HaCaT, with direct-acting genotoxic agents suggest this assay is a reliable non-autoradiographic UDS assay to evaluate the genotoxic potential of a chemical, which has topical exposure risk.
- UV irradiation study in HaCaT cells revealed that long exposure to UVA/B/VIS (≥ 310 nm in wavelength) from a solar simulator cutoff with H1 filter did cause UDS. Furthermore, massive UDS induced by as little as 0.01 J/cm^2 of UVB (≥ 280 nm) from solar simulator through H2 filter raises the concern of skin carcinogenesis under UVB exposure.
- $30 \mu\text{M}$ Aph did hold off the DNA repair and enhanced the FLUDS assay signal.
- All cell lines tested (HaCaT, HepG2, PC3, A431, L929, 3T3, and MDA-MB-157) responded to the UVC irradiation with DNA repair processing (UDS) in quite different manners.
- FLUDS successfully detected chemical-induced UDS in all cell lines (HaCaT, HepG2, PC3, A431, L929, 3T3, and MDA-MB-157).
- FLUDS has several advantages over the standard radiometric UDS assay, specifically
 - ◆ Cost efficiency (less than half the cost)
 - ◆ Shorter time of performance (3 days vs. up to 16 weeks)
 - ◆ Ability to analyze a much larger cell set (5,000+ cells vs. 50-100 per sample)
 - ◆ High throughput 24-well plates allow more compounds to be examined per assay

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