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DNA Damage Signaling Assessed in Individual Cells in Relation to the Cell Cycle Phase and Induction of Apoptosis

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Abstract

Reviewed are the phosphorylation events reporting activation of protein kinases and the key substrates critical for the DNA damage signaling (DDS). These DDS events are detected immunocytochemically using phospho-specific Abs; flow cytometry or image-assisted cytometry provide the means to quantitatively assess them on a cell by cell basis. The multiparameter analysis of the data is used to correlate these events with each other and relate to the cell cycle phase, DNA replication and induction of apoptosis. Expression of γ H2AX as a possible marker of induction of DNA double strand breaks is the most widely studied event of DDS. Reviewed are applications of this multiparameter approach to investigate constitutive DDS reporting DNA damage by endogenous oxidants byproducts of oxidative phosphorylation. Also reviewed are its applications to detect and explore mechanisms of DDS induced by variety of exogenous agents targeting DNA such as exogenous oxidants, ionizing radiation, radiomimetic drugs, UV light, DNA topoisomerase I and II inhibitors, DNA crosslinking drugs and variety of environmental genotoxins. Analysis of DDS induced by these agents provides often a wealth of information about mechanism of induction and the type of DNA damage (lesion) and is reviewed in the context of cell cycle phase specificity, DNA replication, and induction of apoptosis or cell senescence. Critically assessed is interpretation of the data as to whether the observed DDS events report induction of a particular type of DNA lesion.

Keywords

ATM; γ H2AX; Chk2; DNA double strand breaks; chromatin relaxation; DNA replication; DNA topoisomerase inhibitors; UV; cytometry; confocal microscopy

I. DNA damage signaling

Damage to DNA triggers a cascade of multifaceted molecular changes collectively defined as the DNA damage signaling (DDS) or DNA damage response (DDR). These DDS events comprise of a multitude of protein post-translational modifications and have four critical goals: (i) enabling accessibility of the DNA repair machinery to the damage site; (ii) activating and engaging repair machinery; (iii) halting cell cycle progression and division to

prevent transfer of damaged DNA to the progeny cells, and (iv) mobilizing mechanisms that prepare cell to undergo either apoptosis or senescence, the means to eradicate cells with extensive DNA damage that cannot be fully repaired (reviews^{1–7}). Flow cytometry and imaging-assisted cytometry are the major methodologies used to assess DDS in individual cells and correlate particular DDS events with the cell cycle phase or induction of apoptosis. This review outlines molecular changes occurring in the course of DDS, describes cytometric approaches detecting these changes, provides examples of their application for mechanistic studies of genotoxic effects of various agents and lists applications of cytometric DDS assays for genotoxicity screening. It updates and expands our prior review which was focused on technical aspects of the methodology.⁸

A. Chromatin relaxation (decondensation)

The chromatin compaction, particularly within regions of heterochromatin, restricts the accessibility of DNA damage sites to DNA damage response proteins making DNA in these regions refractory to repair. Remodeling of chromatin structure that leads to its decondensation (relaxation) is one of the earliest events of the DDR (reviews^{9–13}). This event increases accessibility of the damage sites to the repair machinery. The signal to initiate chromatin decondensation appears to be triggered by decline of torsional strain of the DNA double helical structure resulting from DNA damage, particularly when DNA double-strand breaks (DSBs) are being formed (Fig. 1). In the absence of DNA damage the DNA torsional strain (topological stress) is being maintained by its winding onto the nucleosome core composed of histone octamers causing supercoiling to maintain the supra-nucleosomal chromatin structure.¹⁴ Following DNA damage high mobility group proteins (HMGs) and histone H1 play major role in providing the mechanism for local chromatin decondensation.^{15–18} Compared with other nuclear proteins HMGs are more extensively and rapidly modified by phosphorylation, acetylation, methylation, ribosylation and/or sumoylation in response to a change in the physiological state of the cell, induction of stress or cell cycle perturbation.¹⁹ This set of proteins provides a continuous dynamic interplay modulating molecular structure of chromatin.^{20,21} Of particular significance is the binding of the HMGN1 protein to the nucleosome, which alters the architecture of chromatin and affects the levels of post-transcriptional modifications of the tails of nucleosomal histones. Specifically, upon HMGN1 binding to nucleosomes, phosphorylation of histone H3 on Ser10 is reduced.²⁰ Because Ser10 histone H3 phosphorylation is required to maintain chromatin in a condensed state such as seen during mitosis²² or premature chromosome condensation,²³ its low level of phosphorylation facilitates chromatin decondensation (relaxation). Prevention of histone H3 phosphorylation by activation of HMGN1 and its binding to nucleosomes may thus provide the mechanism for chromatin decondensation in response DNA damage.

Histone acetyltransferase TIP60 also plays significant role in modulation of chromatin spatio-temporal dynamics triggered by DNA damage. It acetylates histone H2AX, which is a prerequisite for its phosphorylation on Ser139. Furthermore, TIP60 regulates the ubiquitination of H2AX via the ubiquitin-conjugating enzyme UBC.^{24,25} The presence of wt p53 appears to be essential for the induction of chromatin decondensation after DNA damage^{9, 26} likely through its effect on the tumor suppressor p33ING2.²⁷ Chromatin relaxation not only augments accessibility of the repair proteins to DNA damage sites but also provides the signal for activation of *Ataxia Telangiectasia Mutated* (ATM) protein kinase through its phosphorylation on Ser1981.

At the moment of chromatin relaxation and ATM activation the MRN complex consisting of Meiotic Recombination 11 Homolog A (Mre11), Rad50 homolog and Nijmegen Breakage Syndrome 1 (NMR1) proteins undergoes translocation into the site of DNA damage.^{28–31} Relaxation of chromatin and translocation of MRN to the damage site are considered both to

contribute to ATM activation. Whereas ATM activation takes place in chromatin at some distance from the DNA break site the activated ATM undergoes rapid translocation to the site.

B. Activation of phosphatidylinositol 3' kinase-related kinases (PIKKs)

The DDR is regulated by three PIKKs: ATM, ATR and Rad3-related (ATR), and DNA dependent protein kinase (DNA-PKcs).^{4,32–34} These kinases phosphorylate multitude of proteins whose ultimate purpose is to preserve integrity of the genome. The substrates phosphorylated by these PIKKs are implicated in regulation of DNA damage repair, cell cycle progression, apoptosis and cell senescence. In many instances these PIKKs can have redundant activities and back-up each other in terms of phosphorylation of the same proteins.

Among the PIKKs activated in response to DNA damage the most extensively studied was ATM, which is the key component of the signal transduction pathways mobilized by the induction of DSBs.^{35,36} Activation of ATM is provided through its autophosphorylation on Ser1981 within the ATM dimer and is preceded by its prior acetylation that is mediated by the Tip60 histone acetyltransferase³⁷. Following ATM phosphorylation the inactive dimers dissociate onto monomers having kinase enzymatic activity.^{2,3} (Fig. 1) The MRN protein complex plays a key role in the process of ATM activation by detecting DSBs, recruiting ATM to the damage site and targeting ATM to the respective substrates to carry on their phosphorylation³⁸. Activation of ATM is also being promoted by p53-binding protein 1 (53BP1)³⁹. The catalytic domain of ATM is outside of the Ser1981 phosphorylated site and becomes accessible to the kinase substrates when ATM is in its monomeric conformation.^{1,2}

It should be stressed that in addition to its essential role in signaling DNA damage ATM can be directly activated by oxidation and plays an important function in oxidative stress response.^{40–42} In this capacity ATM senses redox potential, controls the level of reactive oxygen species within the cell and interacts with the AMP-activated protein kinase (AMPK), mammalian target of rapamycin (mTOR) and hypoxia-inducible factor 1 (HIF1)⁴². Interestingly, unlike in the case of activation by DNA damage which induces monomerization, ATM activated by the oxidative stress remains as a covalently linked dimer.⁴²

As presented in Fig. 1 ATM phosphorylates several substrates at the site of the DSB, including NBS1, Structural Maintenance of Chromosomes 1 (SMC1) and Breast Cancer 1 (BRCA1) proteins. Phosphorylated NBS1 targets ATM towards Chk1, phosphorylated SMC1 engages the S-phase checkpoints halting DNA replication⁴³ and BRCA1 phosphorylation is required to activate this protein along the DNA repair pathway. The BRCA1 (E3-ubiquitin ligase) is involved in several biochemical processes related to DNA repair.^{5,30} BRCA2 is essential for locating Rad51 to the sites of DNA damage and both BRCA proteins are involved in DNA repair by homologous recombination (HR).⁴⁴ Also recruited to the DSB site is the mediator of DNA damage checkpoint 1 (MDC1).⁴⁵ This nuclear protein activates the S phase- and G₂/M phase- cell cycle checkpoints and interacts with phosphorylated histone H2AX near sites of DSB facilitating recruitment of the ATM and other repair factors to the damage site.

Whereas induction of DSBs such as caused by ionizing radiation triggers activation of ATM, DNA damage can also lead to activation of ATR and DNA-PKcs^{32–34}. ATR becomes activated in response to UV radiation and to replication stress e.g. after exposure of cells to inhibitors of DNA polymerase.^{46–48} However, activation of DNA-PKcs takes place during repair of DSBs where it is an essential factor for the non-homologous end-joining (NHEJ) mechanism of DNA repair.^{33,49} Because the NHEJ mechanism also operates during V(D)J

recombination and is responsible for antibody diversity DNA-PKcs is a critical element for normal immune development.⁵⁰ DNA-PKcs is also strongly implicated in telomere maintenance.⁵¹ The process of activation and inactivation of DNA-PKcs is mediated by its extensive post-translational modification³³. Among several sites of its phosphorylation Thr2609, which becomes autophosphorylated in response to DNA damage by ionizing radiation, has been the most extensively studied.⁵²

C. Activation of checkpoint kinases

p53 (TP53), checkpoint kinase 2 (Chk2) and histone H2AX are among the most important downstream substrates phosphorylated by PIKKs.^{2,53} The purpose of checkpoint pathways activation is to halt cell cycle progression until integrity to DNA is restored by the repair mechanisms.^{54–57} In response to induction of DSBs ATM activates Chk2 via phosphorylation of Thr68 of this kinase which leads to Chk2 dimerization and acquirement of the catalytic activity.^{54,55} (Fig. 2) Phosphorylation of Chk2 on Thr68 can also be mediated by ATR, which takes place in response to DNA replication stress.⁵⁶ Further steps in activation of Chk2 involve intermolecular phosphorylation on Thr383, Thr387 and Ser516 within the Chk2 dimers, which leads to dissociation of the dimers. Both the monomers and the multi-phosphorylated dimers are enzymatically active (Fig. 2). The DNA damage-activated Chk2 undergoes dissociation from chromatin which facilitates further signal amplification and translocation to soluble substrates.⁵⁸ Activated Chk2 phosphorylates numerous downstream substrates including Cdc25A and Cdc25C phosphatases which after activation induce cell arrest at the G₁ or at the transition from G₂ to M, respectively (Fig. 2). Chk2 plays also a role in mediating the response to DNA damage by promoting apoptosis. For example after DNA damage induced by the DNA topoisomerase II (Top2) inhibitor etoposide, Chk2 phosphorylates and activates the E2F-1 transcription factor that activates apoptotic pathways.⁵⁹ Phosphorylation of p53 by Chk2 may lead to upregulation of Bax, an event also promoting apoptosis. Phosphorylation of p53 may also lead to upregulation of p21^{Waf1} which in addition to activation of Cdc25A provides another mechanism to stop cell progression through G₁.⁶⁰ Still other substrates phosphorylated by Chk2 are BRCA1 and Promyelocytic Leukemia (PML) proteins.^{61,62} Phosphorylation of BRCA1 engages this protein in the DNA repair pathway while of PML increases cells proclivity to undergo apoptosis.⁵⁵ Activated Chk2 also stabilizes the FoxM1 transcription factor thereby enhancing expression of DNA repair genes.⁶³ There is strong redundancy between Chk1 and Chk2 as well as among all three isoforms of Cdc25 (Cdc25A, Cdc25B and Cdc25C) in their enzymatic activities of phosphorylation (Chk1, Chk2) or dephosphorylation (Cdc25A, Cdc25B, Cdc25C), of the respective substrates.^{64–66} This leads to redundancy and possibility of use different mechanisms to halt cell progression through G₁ or G₂ in response to DNA damage.

D. Histone H2AX phosphorylation

Histone H2AX, one of the variants of histone H2A⁶⁷ plays a critical role in surveillance of genome integrity.^{68–70} In response to DNA damage, particularly when the damage involves induction of DSBs, H2AX becomes phosphorylated on Ser139; so modified H2AX is defined as γ H2AX.^{71–73} The phosphorylation can be mediated by ATM-^{74,75}, ATR-⁷⁶, and/or DNA-PKcs⁷⁷ and takes place on nucleosomes located on both sides flanking DSBs along a megabase domain of DNA.⁷¹ H2AX is also phosphorylated during induction of DSBs in physiological processes such as DNA recombination in V(D)J class-switch during the process of immune system development and in meiosis.^{50,78} DNA fragmentation in cells undergoing apoptosis also induces extensive H2AX phosphorylation.^{79–81}

II. DNA damage signaling (DDS) detected and measured in individual cells

A. Constitutive DDS

In live cells DNA is continuously exposed to reactive oxidants, by-products of aerobic respiration generated in mitochondria, as well as to exogenous oxidants or oxidant-inducers. Such exposure leads to a progressive oxidative DNA damage. According to one estimate during a single cell cycle of 24 h duration in the average cell in the human body the oxidants generate approximately 5,000 DNA single-strand lesions (SSLs).⁸² These lesions are single-strand breaks, apurinic/apyrimidinic sites, oxidation products such as 8-oxoguanine and thymine glycol, and some alkylation products⁸³. Whereas about 99% of SSLs are repaired by essentially error-free mechanisms, one percent of them (~50) become converted to DSBs, predominantly during DNA replication. Recombinatorial repair (homologous recombination repair) and nonhomologous DNA-end joining (NHEJ) are the major pathways for repair of DSBs. The NHEJ pathway is error-prone, often resulting in deletion of a few base pairs.⁸⁴ This leads to an accumulation of DNA damage with each cell division. Such permanent damage if occurs at sites of oncogenes or tumor suppressor genes promotes development of pre-neoplastic changes.⁸⁵ It is also considered to contribute to cell aging and senescence.

The presence of oxidants and certain DNA primary lesions resulting from oxidative damage such as 8-oxoguanine, can be detected in individual cells by cytometry.^{86,87} However, because of the scarcity of DSBs generated by endogenous oxidants their detection is difficult. The comet methodology⁸⁸ lacks the desired sensitivity and it also cannot provide information on the subnuclear location, relationship between the presence of DSBs and the cell cycle phase, or DNA ploidy, of the examined cells.

While studying a variety of cell lines during their unperturbed growth, as well as mitogenically stimulated normal lymphocytes, in the absence of any added exogenous genotoxic agents, we noticed the background level of H2AX phosphorylation and activation of ATM. This background level of γ H2AX expression and ATM phosphorylation was defined as *constitutive DDS*.^{89–93} The level of constitutive DDS was shown to vary between different cell types (cell line) and was higher for cells having wt p53 compared to cells with null- or mutated- p53.⁹³ It also was dissimilar for cells in different phases of the cell cycle being higher for S- and G₂M- compared with G₁- phase cells. Constitutive DNA damage response was markedly attenuated by exposure of cells to N-acetyl-L-cysteine (NAC), the scavenger of reactive oxidative species, and the attenuation was NAC-concentration dependent.⁸⁹ Expression of γ H2AX was also markedly decreased in cells growing at hypoxic (3% O₂) conditions (Fig. 3).

Further studies demonstrated that the decreased metabolic activity such as induced by cell treatment with 2-deoxy-D-glucose or 3-bromopyruvate, exposure to the antioxidants ascorbate or celecoxib, confluency in cultures, as well as growth at low serum concentrations, all reduced the constitutive level of expression of γ H2AX and of activated ATM. Conversely, the elevated level of constitutive expression of γ H2AX and activated ATM was seen to be correlated with the increased metabolic activity and oxidative phosphorylation following mitogenic stimulation of lymphocytes or treatment with dichloroacetate, an agent known to shift metabolism from anaerobic to oxidative glycolysis through its effect on pyruvate dehydrogenase kinase.⁹² It also correlated well with the intracellular level of reactive oxygen species (ROS).⁹¹ Collectively, these data provide strong evidence that analysis of constitutive levels of H2AX phosphorylation and ATM activation in individual cells by multiparameter cytometry offers the means of measuring the effectiveness of factors such as antioxidants, ROS scavengers, caloric restriction mimetics or geroprotecting agents on protection of DNA from the damage caused by endogenous oxidants. Using this cytometric approach the effectiveness of hyaluronate, vitamin D3 and

metformin, the agents considered to have geroprotective properties,^{94–96} in reducing the level of constitutive DNA damage, has been clearly demonstrated.^{97–99}

Interestingly, a strong correlation between the level of constitutive expression of γ H2AX and human age was observed for both males and females, as older individuals had markedly elevated expression of γ H2AX.¹⁰⁰ An increase in constitutive DDS is observed during tumor progression where it appears to provide clinically useful prognostic biomarker of the progression.^{101–105}

B. DDS induced by exogenous oxidants

Exposure of cells to exogenous oxidants triggers DDS. The earliest event of DDS, relaxation (decondensation) of chromatin, can be detected by as early as 10 min after exposure to H_2O_2 .¹⁰⁶ The relaxation is evident in across the cell cycle being approximately of similar degree in G_1 , as in S or G_2M cells. The subsequent events of DDS, however, such as activation of ATM and H2AX phosphorylation, are much more pronounced in S- compared to G_1 - or G_2M - cells (Fig. 4).^{107,108}

As is evident in Fig. 4 the DNA replicating cells marked by the pulse-labeling them with EdU (identified by the red dots in panel D) show considerably higher expression of γ H2AX than the EdU unlabeled cells. A modest degree of correlation ($r = 0.36$) between extent of EdU incorporation and expression of γ H2AX is apparent (Fig. 4D).¹⁰⁸ The extent of EdU incorporation varies between the cells predominantly due to variability in the interval of DNA replication during duration of exposure to EdU (Fig. 4A, en-S and ex-S cells). The correlation thus indicates that the longer the cells were replicating DNA at the time of exposure to EdU (which was constant, 2 h) the greater was the intensity of DDS manifesting as the induction of γ H2AX. These data are consistent with the mechanism in which the elongating replication forks stalled or collapsed upon encountering the primary DNA lesions induced by H_2O_2 provide signal for H2AX phosphorylation.¹⁰⁹

C. DDS induced by ionizing radiation (IR), radiomimetic drugs and UV

The early studies on the detection of DDR in individual cells exposed to IR or radiomimetic drugs were focused on the assessment of a possible relationship between the extent IR-induced γ H2AX, considered to report the presence of DSBs, and cell survival.^{110–116} The induction of γ H2AX in several cell types was detected by flow cytometry already 1 h after exposure to the dose of X-rays as low as a 20 cGy.¹¹¹ While expression of γ H2AX in non-irradiated cells was higher in S-phase the X-ray induced increase in γ H2AX was distinctly more pronounced in G_1 than in S-phase cells.¹¹³ A strong correlation was observed between the induced expression of γ H2AX and cell survival as measured by clonogenicity assays. The half-times of the radiation-induced γ H2AX foci, ranged from 1.6 to 7.2 h, correlated well with clonogenic survival for 10 cell lines.¹¹⁰ Also strongly correlated with cell survival was expression of γ H2AX measured 1 h after treatment of Chinese hamster V79 cells with several radiomimetic drugs examined within a range of over two decades of cell kill.¹¹² This strong correlation led the authors to postulate that the assessment of H2AX phosphorylation by flow cytometry can be used as a surrogate of the cell kill estimate.¹¹² Interestingly, the cell line-dependent differences in the rate of disappearance of IR induced γ H2AX were associated with the status of p53 (wt vs p53 deficient) as well as was related to intrinsic radiosensitivity of the lines.¹¹⁰ The treatment of human and rodent DNA-repair proficient and deficient cell lines with cisplatin revealed that the level of the retention of γ H2AX foci 24 h after the treatment was much more correlated with the fraction of cells that lost their clonogenic potential than the initial intensity of γ H2AX expression following the treatment.¹¹⁶

Several subsequent studies provided further evidence that both, the extent of induction γ H2AX by IR, and perhaps to an even greater degree, the duration (persistence) of the induced γ H2AX, likely reporting the presence of the unrepaired DSBs, are strong biomarkers of cytotoxic effects of IR, predictive of elimination of cells' proliferation capability.^{117–121}

Unlike in the case of IR, the energy of UV light is absorbed by thymine and cytosine (pyrimidine bases) which leads to formation of the four-membered cyclobutane ring of the pyrimidine dimer.¹²² In addition, a single bond may form between two carbon atoms on the cyclobutane ring resulting in the so called '6–4 (T-C) photoproduct'.¹²² Nucleotide excision repair (NER) is the primary mechanism of repair of DNA damage induced by UV.¹²³ One of its earliest events of the DDR is chromatin relaxation. This event, detected by flow cytometry, has been seen in all cells, regardless of the cell cycle phase, already 10 min after exposure to UV.¹⁰⁶

Phosphorylation of H2AX induced by UV is primarily mediated by ATR.^{124,125} However, there is a redundancy and activation of ATM as well as DNA PKcs can also be seen in UV treated cells, contributing to phosphorylation of H2AX and other downstream protein substrates.¹²⁴ Unlike chromatin relaxation which takes place in all phases of the cell cycle,¹⁰⁶ the activation of ATM and induction of γ H2AX by UV was evident exclusively in DNA replicating cells and inhibition of DNA replication by the DNA polymerase inhibitor aphidicolin prevented the induction of γ H2AX.^{126,127} It should be noted, however, that aphidicolin as well other inhibitors of DNA replication such hydroxyurea or excess of thymidine also induce γ H2AX.^{46,47} The cell cycle-related pattern of γ H2AX induction by these inhibitors, however, is very much different than that of the pattern induced by UV. Namely, unlike UV which affects cells uniformly across the S-phase, the most affected by the replication inhibitors were cells initiating DNA replication, at the very-early section of S-phase.^{46,47}

Further evidence linking DNA replication with the induction of γ H2AX by UV was obtained from the studies designed to correlate incorporation the DNA precursor 5-ethynyl-2'-deoxyuridine (EdU) with the extent of induction of γ H2AX in the same cells. Exposure of cells to EdU to mark the cells replicating DNA, followed by exposure to UV revealed that γ H2AX was induced *exclusively* in EdU-positive cells, and the extent of EdU incorporation versus the level of UV-induced γ H2AX were strongly correlated ($R^2 = 0.98$).¹²⁷ There was no evidence of H2AX phosphorylation in the non-replicating cells.¹²⁷ The data are consistent with the mechanism in which the UV-induced primary DNA lesions cause an initial total block to replication forks elongation.^{128–130} However DNA replication may still continue leaving unreplicated gaps behind the replication forks. It is not entirely clear to what extent the formation of DSBs¹³⁰ versus the block in forks elongation^{128,129} contribute to activation of ATR, ATM and DNA-PKcs that leads to H2AX phosphorylation. It should be noted that the H2AX phosphorylation may occur during replication stress in the absence of DSBs formation,⁸¹ and thus the γ H2AX may not be induced exclusively in response to DSBs caused by UV. Definitely, however, the induction of γ H2X takes place only during replication.

D. DDS induced by anticancer drugs targeting DNA

1. DNA topoisomerase inhibitors—DNA topoisomerase I and II (Top1, Top2) inhibitors are among the most widely used anticancer drugs. Their mechanism of action is considered to involve stabilization of Top-DNA complexes ("cleavable complexes") which are transiently formed to cut DNA in order to untangle double helix which becomes entangled or over-wound during replication or transcription. Collisions of elongating replication forks, or moving RNA polymerase molecules, with these complexes result in

formation of DSBs, the potentially lethal lesions.^{131–133} Extensive studies were carried out to compare the cells cycle-specific effects of Top1 inhibitor topotecan (Tpt) with the Top2 inhibitors mitoxantrone (Mxt) and etoposide (Etp).^{134–142} Tpt is the analog of camptothecin (Cpt), binds to DNA by intercalation and stabilizes the Top1-DNA complex.¹⁴³ The Top2 inhibitor Mxt which is anthracenedione analog also intercalates into DNA^{144,145} and stabilizes Top2-DNA complex.¹³³ Etp (also named VP-16), one of the podophyllotoxins family of drugs, binds to the Top2-DNA complex stabilizing it.¹⁴⁶ The pattern of DDS as related to the cell cycle phase specificity, induction of apoptosis and involvement of ROS was very much different for each drug.^{134–140}

Specifically, the treatment of human lymphoblastoid TK6 or pulmonary adenocarcinoma A549 cells with Tpt (or Cpt) led to rapid induction of phosphorylation of ATM and H2AX exclusively in S phase cells. In contrast, their exposure to Mxt or Etp induced ATM and H2AX phosphorylation in all phases of the cycle.^{134,136,138} Moreover, whereas DDS induced by Mxt or Etp was significantly attenuated by the reactive oxygen species (ROS) scavenger N-acetyl-L-cysteine (NAC), no attenuation at all was seen after induction of DNA damage by Tpt or Cpt.^{135,138} The induction of apoptosis, seen subsequently to ATM activation and H2AX phosphorylation, was also cell cycle-phase specific. The S-phase cells were selectively undergoing apoptosis in cultures treated either with Tpt/Cpt or Mxt whereas apoptosis of the cells treated with Etp was seen in all phases of the cell cycle and actually was more pronounced in G₁ phase.¹³⁴

A relationship between the induction of DDR by Tpt, Mxt and Etp and actual DNA replication was explored by multiparameter cytometry, by labeling DNA replicating cells with EdU and measuring the induction of γ H2AX, differentially labeled, in the same cells (Fig. 5).¹⁴⁷ Toward this end the topoisomerase inhibitors were included into the cultures at the time when the cells were incorporating EdU. The multiparameter cytometric analysis made it possible to identify EdU-incorporating cells and using a “*paint-a-gate*” algorithm to directly correlate the expression of γ H2AX with EdU incorporation, on cell by cell basis.¹⁴⁷ As is evident in Fig. 5 the induction of γ H2AX after exposure to Cpt is seen exclusively in DNA replicating cells. Also, a relatively strong correlation between EdU incorporation and induction of γ H2AX is evident in cells treated with Cpt ($r = 0.86$). In cells treated with Mxt or Etp, on the other hand, both DNA replicating as well as non-replicating cells demonstrate distinctly elevated level of γ H2AX. The correlation between induction of γ H2AX and EdU incorporation is also less apparent (Mxt, $r = 0.52$; Etp, $r = 0.64$). The data shown in this figure also indicate that, as would be expected, the treatment with Cpt, Mxt, or Etp decreased DNA replication rate revealed by lowered intensity of labeling with EdU seen in the D, G, and J panels compared with A (Fig. 5).

The approach to correlate actual DNA replication sites with the induction of γ H2AX, has been further advanced using confocal microscopy to measure the spatial relationship in chromatin between the sites of DNA replication (“replication factories”) and the sites of γ H2AX foci, the latter considered to be reporters of the induction of DSBs.^{72,73} Toward this end the cells were shortly exposed to EdU and subsequently treated with Tpt, Mxt, or Etp (Fig. 6).¹⁴⁷ The incorporated EdU was detected using the green-fluorochrome (AlexaFluor 488)-tagged azide, whereas the Tpt-, Mxt-, or Etp- induced γ H2AX foci were detected immunocytochemically using Ab conjugated to a red fluorescing dye (AlexaFluor 568). Among the DNA replicating cells the greatest degree of co-localization of γ H2AX foci with DNA replication sites was seen in Tpt-treated cells, particularly during early-S ($R > G = 0.66$). The co-localization was also quite evident in the early-S phase Mxt-treated cells ($R > G = 0.42$) but was very low in late-S phase cells ($R > G = 0.02$). Etp-treated cells showed relatively low level of co-localization in both, early- ($R > G = 0.16$) and late-S phase ($R > G = 0.19$) phase cells.

All these studies employing cytometry and confocal microscopy provided wealth of information regarding cell cycle phase differences in the mechanism by which these DNA topoisomerase inhibitors induce DDS and subsequently apoptosis. Specifically, the effects of Tpt/Cpt can be explained exclusively by stabilization of DNA-Top1 cleavable complexes. As originally postulated, the collisions of replication forks with these complexes generate DSBs.^{131–133} Rapid (10–30 min) ATM phosphorylation and induction of γ H2AX exclusively in S-phase cells, at the sites of DNA replication factories (Fig. 6), is followed 2 h by Chk2 activation and 4 h by p53-Ser15 phosphorylation, all preferentially in S-phase cells.^{141,142} Apoptosis is also induced exclusively in S-phase cells.^{79,80} All these findings are consistent with the mechanism in which collisions of replication forks with the stabilized DNA-Top1 complexes are the sole mechanism of induction of DDS and subsequent apoptosis. The lack of evidence of DDS in G₁ or G₂M cells points out that collisions of the moving RNA polymerase with DNA-Top1 complexes during translation¹³¹ play no role in the possible induction of DSBs. Also, there is no involvement of ROS.^{135,138}

More complex is cell response to Mxt. While ATM activation and H2AX phosphorylation was seen in all phases of the cell cycle, the S-phase cells were preferentially undergoing apoptosis.^{80,141} Thus, collisions of replication forks with the stabilized DNA-Top2 complexes, which as evident from colocalization of replications sites and γ H2AX foci (Fig. 6) appear to be much more extensive during the early- than the late- S phase (Fig. 6), seem to be responsible for induction of γ H2AX and apoptosis. However, significant involvement of ROS in the induction of DDS (ATM, Chk2, p53 and H2AX phosphorylation) was also apparent in as much as the ROS scavenger NAC markedly suppressed DDS events, in all phases of the cell cycle.^{134,135} In the case of Etp, the collisions of replicating forks with the DNA-Top2 complexes seem to play relatively minor role, as evident with low level of co-localization of γ H2AX (DSBs) and DNA replication sites (Fig. 6). Similar to Mxt, the involvement of ROS contributes to the induction of DDS in all phases of the cell cycle in cells treated with Etp. Because of DDS seen in G₁ and G₂M cells the possible role of RNA polymerase molecules colliding during translation with the stabilized DNA-Top2 complexes cannot be excluded as an additional factor contributing to the induction of DDS in cells treated with Mxt or Etp.

The induction of γ H2AX and activation of ATM was detected in peripheral blood blasts of the patients with different types of acute leukemia treated with topoisomerase inhibitors, one hour after drug infusion. The number of the studied patients (20) and the number of the clinically non-responding ones (2) was too low to draw a conclusion whether the extent of treatment-induced DDS can be clinically prognostic.¹⁴⁹ However, the findings demonstrate the feasibility of assessment of DDS during treatment of leukemia with drugs targeting DNA and its possible use as biomarker predictive of treatment effectiveness.¹⁴⁹

2. DNA crosslinking agents—Exposure of cells to DNA crosslinking agents such as cisplatin or mitomycin C triggers DDS, revealed as induction of γ H2AX, predominantly in S-phase cells.^{80,116,150–152} Compared to DNA topoisomerase inhibitors the kinetics of γ H2AX induction by these agents is different. Unlike in the case of Mxt or Tpt which induce γ H2AX already 10 min after cell exposure, the rise in expression of γ H2AX occurs with a 2–3 h delay and is seen in all phases of the cell cycle.⁸⁰ The repair process of DNA damage by cisplatin involves the NER mechanism, known to generate ssDNA breaks. A fraction of ssDNA lesions is known to be converted to DSBs. Furthermore, repair of cisplatin-induced damage also involves NHEJ.⁸⁰ This mechanism may additionally contribute to the formation of ds DNA breaks and to the observed H2AX phosphorylation. Thus, when the primary cisplatin-induced lesions do not involve ds DNA breaks, but ds DNA breaks are formed during DNA repair, H2AX phosphorylation may indicate efficiency of the repair process. This mechanism is consistent with the observation that persistence of

γ H2AX foci, most likely reporting unrepaired DNA, is predictive of the cell-lethal effects of the treatment.¹⁵² It has been postulated that assessment of frequency of cells that retain γ H2AX foci 24 h after treatment may predict tumor cell killing.¹⁵²

Exposure of cells to very low concentrations of mitomycin C for a prolonged time period (6 days) was shown to trigger moderate level of DDS manifesting as elevation of γ H2AX expression.¹⁵⁰ Such treatment induced cell senescence and irreversible loss of reproductive capacity. Mutations of oncogenes and tumor suppressor genes that lead to activation of mTOR signaling are common to cancer. Because mTOR activation predisposes cells to undergo senescence¹⁵³ it was postulated that cancers characterized by such mutations may be particularly sensitive to treatment with low doses of DNA damaging drugs (“metronomic chemotherapy”) that cause persistent replication stress.¹⁵⁰ Analysis of DDS in response to persistent replication stress is found to be predictive of cell senescence may provide the means to identify tumors prone to undergo senescence when treated with low drug doses.

E. DDS as a marker of genotoxicity. Screening for environmental genotoxins

High sensitivity in detection of DNA damage combined with a possibility to correlate it with the cell cycle phase and/or apoptosis as offered by flow or imaging cytometry, contributed to wide application of this approach for screening of a variety of agents with respect to their genotoxic properties. One of first such applications was screening for genotoxic effects of cigarette smoke^{154–160}. The induction of γ H2AX foci, considered to be markers of DSBs, after exposure of normal human bronchial epithelial cells *in vitro* to cigarette smoke or smoke condensate, seen already 10 min after the exposure, was proposed as a new assay for carcinogens in tobacco.¹⁵⁴ The induction γ H2AX, measured by laser scanning cytometry, did not show significant differences vis-à-vis cell cycle phase.¹⁵⁷ (Fig. 7) Activation of ATM and other events of DDS paralleled H2AX phosphorylation.¹⁵⁶ Human lung adenocarcinoma A549 cells, responded to cigarette smoke similarly as normal bronchial epithelial cells thereby providing an alternative experimental model for screening tobacco products.^{155–157} Interestingly, the smoke from tobacco-free cigarettes, which are commercially available as a substitute for tobacco cigarettes (with the aim to curtail smoking) were more potent in inducing γ H2AX and ATM-S1981P, and thus potentially more carcinogenic, than tobacco cigarettes.¹⁵⁹ (Fig.7). The induction of DDS by cigarettes smoke is prevented to a large degree by cells’ exposure to the ROS scavenger NAC.¹⁶¹ (Fig. 7) It is likely thus, that ROS mediate some of the genotoxic effects of tobacco smoke. Cigarette side-stream smoke was reported to induce DDS¹⁵⁸ thereby confirming reports on genotoxic properties of secondhand smoke.¹⁶²

As exemplified by analysis of genotoxicity of cigarette smoke, the cytometric analysis of DDS, particularly of γ H2AX, provides high sensitivity, convenience and rapidity in detecting the genotoxic potential of different agents. This approach was recently used to assess genotoxicity of different types of nanoparticles.^{163–166} Of interest was the observation that the orthopedic particles made of metal (CoCr alloy) induced high level of γ H2AX whereas the ceramic particles had no such effect.¹⁶⁶ This observation provides further evidence of the advantage of the ceramic over metal alloys implants. Possible involvement of reactive oxidants in the induction of DDS by different types of nano- or micro-particles was a subject of particular inquiry in several studies.^{163–166}

Utility of the γ H2AX assay has been also tested in the assessment of genotoxic properties of agents used to manufacture plastics and epoxy resins such as bisphenol A and bisphenol F,¹⁶⁷ ubiquitous pollutants such as polycyclic aromatic hydrocarbons,¹⁶⁸ the non-ionic surfactants nonylphenol polyethoxylates that are widely used for industrial and household purposes,¹⁶⁹ potential genotoxins in cosmetic preservatives,¹⁷⁰ or several different pharmaceuticals frequently detected in freshwater environment.¹⁷¹

The finding that the nitrogen oxide-releasing aspirin induces γ H2AX, activates ATM and leads to apoptosis of TK6 cells provides another example of detection of genotoxic effects by assessing induction of DDS.¹⁷² This is an important observation since so modified aspirin, which as it appears from these findings may have genotoxic properties, is being promoted as a novel anti-inflammatory agent. Attenuation of genotoxicity of the acridine orange mutagen 191 by chlorophyllin, likely *via* the mutagen-interceptor mechanism¹⁷³ was also detected using the γ H2AX assay.¹⁷⁴ In a yet another application of the DDS assay it was observed that exposure of cells to Hoechst 33342 or DRAQ5 fluorochromes, the widely used supravital DNA probes, led to activation of ATM and Chk2 as well as phosphorylation of p53 and H2AX.¹⁷⁵ This observation cautions on use of these probes, which themselves appear to have DNA damaging properties, in long-term cell viability studies.

The successful use of DDS assays for screening and examining genotoxic properties of different agents prompted several authors to propose this approach as the end-point for the assessment of genotoxicity^{176–178}. Developments and applications of the γ H2AX assay in the areas of radiation bio-dosimetry, evaluation or validation of new cancer drugs in clinical studies, chronic inflammation and environmental genotoxicity, have been recently highlighted in comprehensive and authoritative reviews.^{178,179}

III. New cytometric techniques and approaches for analysis of DDS

Cytometry remains a vibrant and dynamic field of engineering where new technological advancements often lead to development of new bioassays probing molecular mechanisms in individual cells.^{180,181} In the recent years in particular we have seen many spectacular developments in the field of cytometry.^{180,181} The latest innovative platforms such as multispectral imaging cytometry^{182–184}, mass cytometry^{185–188}, Raman cytometry^{189–191}, and microfluidic Lab-on-a-Chip^{192–195} solutions rapidly emerge as the next generation of tools for the analysis of DDS.

The multispectral imaging cytometry (Image Stream Flow Cytometry) revolutionized the conventional flow cytometry by integrating a fast in-flow imaging with the conventional layout of the flow cytometer.^{182–183} Such hybrid cytometric system is capable of simultaneously acquiring six spectrally decomposed images from each cell providing a valuable morphometric analysis.^{182–183} Multispectral cytometry has recently revealed distinct frequencies of γ -H2AX foci induction in DNA double strand break repair defective human cell lines.¹⁸⁴

Mass cytometry is yet another proverbial quantum leap in expansion of analytical capabilities probing individual cells.^{185–187} Instead of using fluorescent probes cell staining is performed with stable isotopes of rare earth metals. Mass cytometry evades limitations of conventional flow cytometry such as the need for compensation of the emission spectra and offers a possibility for multiparametric analysis supreme to any other methodology currently available on the market.^{185,186} It has already been shown in the analysis of the cell cycle with the pRb phosphorylation [p-Rb(S807/S811)] as a biomarker.¹⁸⁷ Moreover, mass cytometry it has been also shown capable of discriminating live vs. dying cells using platinum-containing chemotherapy drug cisplatin.¹⁸⁸

Raman Spectral Flow Cytometry is another example of enabling bioanalytical technology of recent years.^{189–190} Non-resonant Raman spectroscopy is one of the most dynamically developing technologies based on inelastic scattering of incident photons and resulting vibrational excitation of the biomolecules.^{181–182} Non-invasive methods of detecting necrotic cell death using Raman Cytometry has recently been reported. Also the Raman micro-spectroscopy to non-invasively track cell cycle dynamics in living cells has also been recently investigated.¹⁸⁹ The advent of surface enhanced Raman spectroscopy (SERS),

which features signal enhancement of over 14 orders of magnitude over non-enhanced Raman scattering, opens up new avenues for real-time dynamic spectroscopy.^{180,190} Nanostructures within a hybrid SERS probe provide sufficient enhancement in the electromagnetic field to analyze single biomolecular species while using only low laser powers and short data acquisition times.^{180,190,191} The SERS flow cytometry was recently developed providing prospects of a massively multiplexed chemical cytometry on living cells.¹⁹¹

Capacitance cytometry is also a radical and pioneering approach to develop label-less flow cytometry.¹⁹⁶ This technology is based on a linear relationship between the DNA content of eukaryotic cell and the change in capacitance that is evoked by the passage of individual cell across a 1-kHz electric field.¹⁹⁶ It has been capable of rapid analysis of cell cycle and fractional DNA content (sub-G₁) without a necessity to label or process cell samples.¹⁹⁶ A capacitance flow cytometry can be particularly useful in the real-time monitoring of changes in cell cycle distribution offering non-exciting analytical advantages.¹⁹⁶

The image-based cytometry represents a useful alternative to flow cytometry as it combines advantages of flow cytometry (FCM) and fluorescence image analysis (FIA).^{181,197} Automated fluorescence mosaic microscopy as a high-content imaging cytometry has recently been proven in systematic analysis of cells and subcellular protein locations such as dose-dependent changes in γ H2AX response.¹⁹⁷ Combined with automated machine learning algorithms it is reportedly a powerful tool for multivariate phenotypic profiling of individual cell nuclei and quantification of subnuclear spots using, optimized image processing tools, and supervised classification.¹⁹⁷

Finally a number of emerging, microfluidic technologies for cell-based assays have recently been reported.^{192–195} The advent of so-called Lab-on-a-Chip technologies (LOC) is one of the most innovative cytometric approaches to the analysis of rare cells and organelles.^{193–194} It also provides exceptional evolutionary avenues for microfluidic flow cytometry (μ FACS), cell sorting and revolutionary cell microarrays.^{181,193,195} Microfluidic flow cytometers and cell sorters require a greatly reduced number of cells per sample when compared with conventional analyzers.¹⁹⁵ A great number of unconventional technologies have recently been proposed for a non-invasive and real-time cell analysis on microfluidic chips. These include real-time studies on a single cell level such as time-of-flight (TOF) optophoresis¹⁹⁸, scanning thermal lens microscopy (TLM)¹⁹⁹ and microfluidic living cell arrays^{192,200}. The microfluidic platforms that can track single cell responses, multiparametrically on a large scale are so far the only tool that can support personalized treatment in oncology and systems biology efforts and provide new vistas for a new generation of rationally designed anti-cancer drugs.^{193,194}

Advances are also being made in analytical chemistry of bioassays. In this regard a high-throughput flow cytometric assays has recently been developed to measure the RPA-ssDNA formation during exposure to DNA damaging agents.²⁰¹ Moreover, a new class of probes for cell imaging, such as functionalized gold or silver nanoparticles has recently been created.¹⁸⁰ The binding of proteins to such nanoparticles changes the wavelength of plasmon resonance, which can be followed by dark-field microscopy.¹⁸⁰ Moreover, the light-scattering intensity of a pair of nanoparticles is greater than for two separate particles, and increases even further for multi-particle assemblies.¹⁸⁰ This feature has been recently utilized to study activation of caspase-3 using crown nanoparticle probes composed of a NeutrAvidin-coated gold-core nanoparticle with biotinylated gold satellite nanoparticles.²⁰² It is postulated that small-cleaved satellite nanoparticles can move and allow analysis of single molecule trajectories.¹⁸⁰ This could be beneficial to track movements of protein complexes involved in DDS.

IV. Interpretation of cytometric data

Detection and evaluation of the extent of DNA damage caused by different genotoxins that would be predictive of potential mutagenic and cytotoxic consequences is the primary goal of most applications of the DDS assays utilizing cytometry. Because DSBs represent the most deleterious DNA lesions, both in terms of their mutagenic potential as well as their role in signaling cell death pathways, it is of importance to correlate the particular events of the DDS with formation of DSBs. Initially, phosphorylation of H2AX on Ser139 was considered to be the specific marker of induction of DSBs. Indeed, in instances of ionizing radiation and radiomimetic agents that directly generate DSBs the intensity of expression of γ H2AX, measured as the integrated γ H2AX phospho-specific immunofluorescence per nucleus correlated well with the extent of DSBs and was prognostic of cell viability.

It should be cautioned, however, that expression of γ H2AX after induction of DSBs is a kinetic event of rather short and often of variable duration. The rate of γ H2AX dephosphorylation varies markedly depending the cell cycle phase, nature of the inducer of DSBs and rate of DNA repair.²⁰³ Expression of γ H2AX however is not measured dynamically, in real time, but at the end-point at the time of cell harvest/fixation. Thus, there is an uncertainty as to whether the expression of γ H2AX is being measured at the peak of response and thus whether intensity of the measured γ H2AX immunofluorescence or frequency of γ H2AX foci correlates with number of DSBs induced by DNA damaging agent.

There is strong evidence that H2AX phosphorylation, triggered by formation of DSBs, is mediated by ATM.^{73,75} One would expect therefore that concurrent ATM activation and induction of γ H2AX may provide more definite evidence of the presence of DSBs than γ H2AX alone. It should be stressed, however, that there are the instances when DDS, including concurrent phosphorylation of both H2AX and ATM, is triggered by DNA lesions other than DSBs. This has been seen to occur at mitosis²⁰⁴ or during premature chromosome condensation.²³ Likewise, ATM and H2AX can be phosphorylated in the absence of DSBs during replication stress.^{46,47,81,204,205} Because of redundancy of PIKKs in phosphorylation of different substrates when one of these kinases is initially activated, activation of the other PIKK often follows. This was observed after induction of DNA damage by UV when activation of ATM was seen to be followed shortly (30 min) after activation of ATR.¹²⁷ Thus, the expression of γ H2AX *per se* or even when accompanied by ATM activation is not a conclusive proof of the presence of DSBs.

The presence of distinct γ H2AX IF foci, each focus reported to represent a single DSB.^{71–73} appears to be more reliable reporter of DSBs. The number of individual IF foci per nucleus, especially if double-differentially labeled with ATM-S1981^P and γ H2AX, is thus likely to correspond to the count of DSBs.^{138,206} Quantification of the foci can be accomplished by image-assisted cytometric analysis utilizing, for example, a laser scanning cytometer (LSC)²⁰⁷ or similar instruments.^{207,209} Multiparameter analysis of such data make it possible to correlate the number of individual foci per nucleus with the cell cycle phase or with a marker of apoptosis.^{8,208} Image analysis was shown to be able to detect very low level of DNA damage, for example, as occurs *in vivo* in lymphocytes of patients subjected to computed tomography examinations.²¹⁰ It should be noted, however, that accurate count of individual foci is limited to their relatively small number (<20) per nucleus. With greater numbers, proximity of foci to each other and possibility of their spatial overlap prevent accurate contouring and counting. Quantification of foci by LSC or other imaging instruments can be accurate, therefore, during analysis of constitutive DDS or for assessing environmental genotoxins at their low concentration, when relatively few foci are present per nucleus.

There are other issues that should also be considered when using image-assisted cytometry for foci quantification. Namely, the rates of phosphorylation and de-phosphorylation of H2AX and/or ATM in individual foci in the same cell may vary depending on the rate of DNA repair at each particular DSB site. Furthermore, when induction of DSBs are asynchronous within the cell (for example as is the case of progressive DNA damage following treatment with low doses of genotoxins) the “age” and thus the IF intensity of individual foci may vary. This variability in intensity of fluorescence of individual foci may complicate the setting optimal thresholds for contouring all foci.

DNA fragmentation mediated by the caspase-activated DNase (CAD) is one of the hallmarks of apoptosis.^{211,212} Fragmentation of DNA triggers massive DDS, reflected by particularly extensive phosphorylation of H2AX.^{79,80} The question may be posed that if apoptosis is induced by agents damaging DNA how DDS triggered by these agents can be distinguished from the secondary DDS triggered by DNA fragmentation induced by CAD. The data shown in Fig. 8 illustrate such an instance, when cells were treated with Tpt which causes DSBs predominantly in S-phase cells, and also induces apoptosis.⁸⁰ As is evident, the level of induction of γ H2AX in apoptotic cells is by an order of magnitude greater than that of γ H2AX induced in response to the primary DNA damage by Tpt. The high intensity of IF of the phospho-specific Ab recognizing phosphorylated H2AX is therefore one of the markers that can be used to identify apoptotic cells and distinguish primary DSBs from apoptotic DNA fragmentation. The other marker is the presence of activated caspase-3 (Fig. 8). It should be noted, however that at late stages of apoptosis γ H2X is being degraded and the intensity of its IF drops.⁸⁰ In still another approach that allows one to identify DDS triggered by the primary DNA damage and exclude apoptosis as the trigger of DNA fragmentation, a caspase inhibitor such as z-VAD-FMK can be used to selectively prevent DNA fragmentation.^{79,80}

Conclusion

The major advantage of cytometry stems from the possibility to correlate expression of particular cell attributes with each other, on cell by cell basis. The correlation analysis is highly informative, often revealing mechanisms of interactions between the measured cell constituents. For many years cytometry has been successfully used for quantitative assessment of different cellular constituents, among them discrete parts of cell signaling pathways. By complementing findings obtained by the methods that rely on data obtained on cells measured in bulk, cytometry contributed a great deal towards revealing molecular mechanisms operating in normal and diseased cells, particularly in areas of cell growth, cell cycle progression and programmed cell death. The introduction of Abs that detect posttranslational modification of proteins, such as phosphorylation, opened an opportunity to explore actual cell signaling along different pathways in individual cells.^{213,214} High sensitivity in detection of DNA damage offered by analysis of γ H2AX foci, approaching the limit of revealing of a single DSB per cell, with a possibility of a measurement of tens of thousands of cells per sample, is one of the major assets of such an approach. As is evident in this review, the multivariate analysis of different cell signaling events along DDS pathway, combined with probes of the cell cycle and apoptosis, yields a wealth of information pertinent to mechanism of action of a variety of genotoxic agents. Further information can be obtained by a combination of high resolution imaging offered by confocal microscopy with multivariate analysis by laser scanning cytometry. This is exemplified by analysis of spatio-temporal relationship between DNA replication and DNA damage sites in revealing mechanism of action of Top1 and Top2 inhibitors (Fig. 5 and 6). It can be expected that approaches along this way will yield further information leading to expansion of our understanding of the mechanisms of DNA damage and DNA repair.

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Abbreviations

53BP1	p53-binding protein 1
Ab	antibody
AMPK	AMP-activated protein kinase
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3-related protein kinase
BRCA1	Breast Cancer 1
CAD	caspase-activated DNase
Chk1	checkpoint kinase 1
Chk2	checkpoint kinase 2
Cpt	camptothecin
DDR	DNA damage response
DDS	DNA damage signaling
DSBs	DNA double-strand breaks
DNA-PKcs	DNA dependent protein kinase
EdU	5-ethynyl-2'-deoxyuridine
Etp	etoposide
FCM	flow cytometry
FIA	Fluorescence image analysis
μFACS	microfluidic flow cytometry
HIF1	hypoxia-inducible factor 1
HMG	high mobility group proteins
HR	homologous recombination
IF	immunofluorescence
IR	ionizing radiation
LSC	laser scanning cytometry
MDC1	mediator of DNA damage checkpoint 1
Mre11	Meiotic Recombination 11 Homolog A
mTOR	mammalian target of rapamycin
Mxt	mitoxantrone

NAC	N-acetyl-L-cysteine
NER	Nucleotide excision repair
NHEJ	non-homologous end-joining
NMR1	Nijmegen Breakage Syndrome 1
PIKKs	phosphatidylinositol 3' kinase-related kinases
PML	Promyelocytic Leukemia protein
ROS	reactive oxygen species
SERS	surface enhanced Raman spectroscopy
SMC1	Structural Maintenance of Chromosomes 1
SSLs	DNA single-strand lesions
TLM	thermal lens microscopy
TOF	time-of-flight
Top1	DNA topoisomerase I
Top2	DNA topoisomerase II
Tpt	topotecan
UBC	ubiquitin-conjugating enzyme

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Fig. 1. The ATM signaling pathway triggered by induction of DSBs.¹⁻⁷

Induction of DSB leads to lessening of torsional strain and unwinding of DNA superhelical structure which triggers local decondensation of chromatin and recruits the MRE11, RAD50 and NBS1 proteins (MRN complex), as well as BRCA1 to the DSB site (A, dashed arrows). These events activate ATM which occurs by autophosphorylation of Ser1981 and leads to dissociation of the ATM dimer onto two monomers that have enzymatic activity. Activated ATM is then recruited to the site of the DSB (B, dashed arrow) where it phosphorylates several substrates including NBS1, BRCA1 and SMC1 (C). NBS1 phosphorylation is required for targeting ATM to phosphorylate Chk1 and Chk2. Phosphorylation of SMC1 activates S-phase checkpoints whereas BRCA1 phosphorylation engages this protein in the DSB repair pathway. ATM also phosphorylates E2F1, Chk1, p53, Mdm2, Chk2, and H2AX and several other substrates. Activated p53 (phosphorylated on Ser 15) induces transcription of p21^{WAF1} and/or Bax genes whose protein products arrest cells in G₁ or promote apoptosis, respectively.

Fig. 2. Activation of Chk2 and Chk2's major substrates.^{54–66}

DNA damage (induction of DSBs) triggers activation of ATM (Fig 1) which in turn phosphorylates Chk2 on *Thr68* causing its dimerization. In response to replication stress rather than DSB Chk2 can be phosphorylated by ATR. Within the dimer Chk2 is further phosphorylated at *Thr383*, *Thr387* and *Ser516* which leads to its dissociation onto monomers. Both multi-phosphorylated dimers and monomers are enzymatically active and able to phosphorylate the downstream substrates. Among these substrates are the Cdc25C and Cdc25A phosphatases whose phosphorylation by Chk2 promotes binding to a 14-3-3 protein thereby preventing translocation into the nucleus and dephosphorylation of inhibitory phosphorylation at *Thr14* and *Tyr15* on cyclin/CDK complexes. This halts cell cycle transitions from G₂ to M (Cdc25C) and G₁ to S, (Cdc25A) respectively. Phosphorylation of Cdc25 phosphatases also accelerates their proteasomal degradation. A redundant mechanism of cell arrest in G₁ involves phosphorylation of p53 by Chk2 which may lead to upregulation of the cdk2 inhibitor p21^{CIP1/WAF1}. Phosphorylation of p53 may also result in upregulation of the pro-apoptotic protein Bax. Apoptosis may additionally be promoted by phosphorylation of PML and E2F-1. Phosphorylation of BRCA1 engages it in the DNA repair pathway.

Fig. 3. Attenuation of constitutive expression of γ H2AX by treatment with the ROS scavenger N-acetyl-L-cysteine (NAC) (A) and by hypoxia (B)

A. The bivariate (DNA content versus γ H2AX IF) distributions show a decrease in the level of constitutive expression of γ H2AX in TK6 lymphoblastoid cells growing in the presence of 10 or 50 mM NAC, added into cultures for 1 h prior to cell harvesting, compared to the untreated cells (Ctrl). The percent decline in mean values of γ H2AX IF of G₁, S and G₂M phase cell subpopulations in cultures treated with NAC in relation to the respective subpopulations of the untreated (Ctrl) cells are shown above the respective arrows. The inset in the left panel shows the DNA content frequency histogram representative of the cells in these cultures. The right panel shows the plot of the mean values of γ H2AX IF for G₁, S and G₂M cells, established by gating analysis, in relation to NAC concentration⁹⁰. Because the level of constitutive expression of γ H2AX (or ATM-S1981P) is relatively low, compared with the level seen after treatment with DNA damaging agents (e.g. Fig. 4) the γ H2AX IF measurements have been carried out at higher sensitivity (higher voltage) of the photomultiplier.

B. Constitutive expression of γ H2AX in WI-38 cells growing in 21% concentration of O₂ (a), and 3% concentration of O₂ for 24 (b) and 48 h (c).⁹².

Fig. 4. Correlation between DNA replication and induction of γ H2AX in A549 cells treated with H_2O_2

A: Incorporation of EdU by untreated cells during 120 min exposure to EdU. The cells which during the duration of 120 min EdU pulse were entering (en-S) or exiting (ex-S) S phase show variable level of EdU incorporation as the interval of their exposure to the precursor at the time of DNA replication varied; the mid-S phase cells show maximal EdU incorporation. **B:** The cells were initially treated with EdU for 60 min and then, still in the presence of EdU, were exposed to 200 μ M H_2O_2 for an additional 60 min. Note a decline in EdU incorporation compared to A. For the “*paint-a-gate*” multiparameter analysis the cells incorporating EdU (above the threshold marked by the dashed line), were electronically colored red. **C:** Constitutive expression of γ H2AX in untreated (control) cells; the skewed dashed line shows the upper threshold of γ H2AX expression for 97% of G_1 and S-phase cells. **D:** Cells were treated as in B, the EdU incorporating cells are colored red. Note the increased expression of γ H2AX (compared with C) predominantly in DNA replicating cells. **E:** Bivariate (EdU vs γ H2AX) distribution shows a correlation between incorporation of EdU and expression of γ H2AX. The EdU negative cells expressing γ H2AX (arrow) are predominantly G_2 M cells that show increased level of constitutive DNA damage signaling.⁹⁸

Fig. 5. Correlation between induction of H2AX by Cpt, Mxt, and Etp and DNA replication in A549 cells

Exponentially growing A549 cells were exposed to EdU for 30 min and then (still in the presence of EdU) were treated either with Cpt (D, E, F), Mxt (G, H, I), or Etp (J, K, L) for 2 h. Control cells (A—C) were exposed only to EdU for 2.5 h. The cells were then fixed and their blue (DAPI), green (EdU), and red (γ H2AX) fluorescence were measured by LSC.¹⁴⁷ The cells that incorporated EdU were “paint-gated” (red) and the data were plotted as bivariate distributions representing γ H2AX versus DNA content (B, E, H, K) or γ H2AX versus EdU incorporation (C, F, I, L). Note that Cpt induced γ H2AX only in EdU incorporating cells. The very few EdU-negative cells expressing γ H2AX, marked by the arrows, (F) are predominantly G₂M cells constitutively expressing γ H2AX (C). In contrast, in response to treatment with Mxt or Etp, H2AX was phosphorylated in both EdU incorporating and non-incorporating (G₁ and G₂M) cells. A correlation between EdU

incorporation and induction of γ H2AX by the Top inhibitors is shown in Panels F, I, and L; the correlation coefficient refers only to DNA replicating cells. Unlike in the case of Cpt-treatment, there are numerous EdU-negative cells expressing γ H2AX following exposure to Mxt or Etp (I, L, marked by arrows). Insets in A, D, G, and J panels present DNA content frequency histograms from the respective cultures, with EdU incorporating cells marked in red. The dashed skewed lines in B, E, H, and K show the upper threshold for 97% of G₁ and S-phase cells expressing γ H2AX in the untreated culture (Ctrl).¹⁴⁷

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Fig. 6. Spatial relationship in chromatin between the sites of EdU incorporation (“replication factories”) and the induction of γ H2AX foci in A549 cells treated with Tpt, Mxt or Etp
Confocal images of A549 nuclei that were briefly (30 min) exposed to EdU and then for additional 60 min treated with Tpt, Mxt, or Etp as described in detail elsewhere.¹⁴⁷ The incorporation of EdU was detected utilizing AlexaFluor 488-tagged azide (green fluorescence) while γ H2AX was detected using a secondary Ab labeled with AlexaFluor 568 (red fluorescence), as in Fig. 5. The size marker = 10 μ m. The top row shows nuclei of cells not incorporating EdU, the mid row - nuclei of cells in early-S phase, the bottom - nuclei of late-S phase. The distinction between early- and late-S phases is based on the characteristic differences in the pattern of DNA replication sites¹⁴⁸. Enlarged images of

selected foci/sites of DNA replication (late-S phase) are shown at the bottom of the respective columns; the size marker = 0.5 μ M. The fraction of DNA replication sites that co-localize with γ H2AX foci (yellow fluorescence) is shown in each panel.¹⁴⁷

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Fig. 7. Induction of γ H2AX in A549 cells after their exposure to cigarette smoke

A. Bivariate (DNA content *versus* γ H2AX) distributions representing mock-treated (Ctrl), cells exposed to smoke from standard tobacco cigarette (2R4F) (TCS), or smoke from tobacco-free (Bravo) cigarette (NTCS). A549 cells were exposed to the smoke for 10 min and the induction of γ H2AX was assessed 1 h after the exposure¹⁵⁹. The skewed dashed line shows the top level of γ H2AX expression for 97% cells from Ctrl culture.

B. Reduction of the cigarette smoke induced γ H2AX in A549 cells by co-treatment with the radicals' scavenger n-acetyl-L-cysteine (NAC); p<0.01 marked by asterisks.¹⁶¹

Fig. 8. Correlation between induction of γ H2AX, activation of caspase-3, and cell cycle phase of HL-60 cells treated with Tpt for 3 h

After 3 h of treatment with Tpt some cells (predominantly in S phase) undergo apoptosis. Apoptotic cells (Ap) show an order of magnitude higher intensity of γ H2AX phospho-specific IF compared with non-apoptotic cells. The caspase-3 *versus* γ H2AX bivariate distributions (right panels) reveal that some non-apoptotic (caspase-3 negative) cells have elevated expression of γ H2AX reflecting the presence to the Tpt-induced primary DSBs (pDSBs).⁸⁰