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Cell synchronization by inhibitors of DNA replication induces replication stress and DNA damage response: analysis by flow cytometry

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Abstract

Cell synchronization is often achieved by inhibition of DNA replication. The cells cultured in the presence of such inhibitors as hydroxyurea, aphidicolin or thymidine become arrested at the entrance to S-phase and upon release from the block they synchronously progress through S, G₂ and M. We recently reported that exposure of cells to these inhibitors at concentrations commonly used to synchronize cell populations led to phosphorylation of histone H2AX on Ser139 (induction of γ H2AX) through activation of *ataxia telangiectasia mutated* and *Rad3-related* protein kinase (ATR). These findings imply that the induction of DNA replication stress by these inhibitors activates the DNA damage response cell signaling pathways and caution about interpreting data obtained with the use of cells synchronized such way as representing unperturbed cells. The protocol presented in this chapter describes the methodology of assessment of phosphorylation of histone H2AX-Ser139, ATM/ATR substrate on Ser/Thr at SQ/TQ cluster domains as well as *ataxia telangiectasia mutated* (ATM) protein kinase in cells treated with inhibitors of DNA replication. Phosphorylation of these proteins is detected in individual cell immunocytochemically with phospho-specific Ab and measured by flow cytometry. Concurrent measurement of cellular DNA content and phosphorylated proteins followed by multiparameter cytometric analysis allows one to correlate extent of their phosphorylation with cell cycle phase.

Keywords

DNA repair; DNA double-strand breaks; flow cytometry; apoptosis; DNA fragmentation; G₁/S boundary

1. Introduction

1.1. Virtues and vices of different synchronization methods

Different approaches are being used to obtain populations of cells synchronized in the cell cycle (*reviews*: 1–4). Each of these approaches offers some advantages but also suffers limitations. One of the widely used methods is based on isolation of mitotic cells by their detachment from flasks during culturing (5). Its virtue stems from the fact that the cell progression through the cycle is not being perturbed. However, the technique is applicable to few cell lines, such as Chinese hamster ovary (CHO) or HeLa cells, that grow attached in cultures and detach during mitosis. Another technique selecting mitotic cells was designed for murine leukemic L1210 cells that grow in suspension but are being enforced to attach to

polylysine-coated membranes. During mitosis the daughter cells detach from the mother cells, still attached to membranes, and float in culture medium (6,7). It is unknown how widely this approach can be used because cells of other than L1210 lines cannot be synchronized this way (8). Cells of certain lines can be synchronized in G₁ by inhibitors of protein farnesylation and geranyl-geranylation such as statins (9), or CDK2 protein kinase inhibitors (10–12). These techniques also are not universally applicable since many lines do not respond to statins or protein kinase inhibitors by reversible arrest in G₁.

Normal, non-tumor cells can be synchronized in G₀/G₁ by removal of growth factors e.g. by “serum starvation” (13), or depletion of a particular amino-acid (14), or by contact inhibition (15). These approaches generally fail to synchronize tumor cell lines. Moreover, the metabolism of so synchronized cells is often perturbed which affects their rate of progression through the cycle upon release from the arrest (16). It should also be noted that the cells synchronized in early G₁ or at mitosis start to lose synchrony while progressing through G₁ and thereby become less synchronous in S or G₂.

The approach based on separation of cells based on their size, centrifugal elutriation, is also being used to obtain relatively synchronous cell populations (17). However, while this procedure does not perturb cell cycle progression the synchrony of uniformly sized elutriated cells is not always sufficiently narrow. Furthermore, elutriation requires rather complex and expensive instrumentation and an experienced operator. Density gradient centrifugation yields cell populations even less synchronized than the elutriation (18). Fluorescence-activated cell sorting (FACS) is also used to obtain synchronous cell populations e.g. based on DNA content analysis upon supravital staining with fluorochromes such as Hoechst 33342 (19). However, Hoechst 33342 elicits DNA damage response (20), long-term toxicity (21), and undergoes redistribution from labeled to unlabeled cells in mixed cell populations (22).

Cell synchronization at mitosis by mitotic spindle poisons such as colchicines, vinca alkaloids or nocodazole is another common approach (23,24). The advantage of this approach is simplicity, low cost and high degree of synchrony when one opts to obtain mitotic or immediately postmitotic cell populations (25). As mentioned, so synchronized cell populations become less synchronous after progression through G₁. Furthermore, undesirable effects such as cytotoxicity and growth imbalance are seen during arrest in mitosis (26–28).

1.2. Inhibitors of DNA replication

Among the most common approaches to obtain populations of synchronized cells, particularly in S-phase, is cell synchronization with the use of DNA replication inhibitors such as hydroxyurea, methotrexate, aphidicolin or high concentration of thymidine (29–32). A combination of replication inhibitors with other synchronizing agents is also widely used (24,33). The advantage of cell synchronization by DNA replication inhibitors is the simplicity and low cost. However, the major drawback is the induction of growth imbalance (26,34). While cells become arrested in their progression through S, their growth, in terms of RNA and protein synthesis, and cell enlargement continues which leads to perturbation of metabolic functions. Furthermore the cell cycle progression machinery of cells synchronized by DNA replication inhibitors is severely perturbed as reflected by unscheduled expression of cyclin proteins (35).

Inhibitors of DNA replication such as hydroxyurea, aphidicolin, thymidine and aphidicolin were shown to induce phosphorylation of histone H2AX on Ser-139 (36,37). Phosphorylated H2AX is defined as γ H2AX (38). Phosphorylation of H2AX and activation of ataxia telangiectasia mutated protein kinase (ATM) through its phosphorylation on Ser 1981

(ATM-S1981^P) are the key markers signaling DNA damage response (DDR) reflecting DNA damage that involves formation of DNA double-strand breaks (DSBs) (38-39; *reviews* 40-41). Both, activation of ATM and H2AX phosphorylation can be detected immunocytochemically using phospho-specific Abs, and the extent of their phosphorylation reporting severity of DNA damage can be measured by flow or laser scanning cytometry (42-46). Since during replication stress induced by inhibitors of DNA replication H2AX is phosphorylated by ATM Rad3-related protein kinase (ATR) and not by ATM, the latter kinase remains not activated (36; *see* Fig 1). While no phospho-specific Ab are available at present to directly detect activated ATR its activation can be detected indirectly using Ab to ATM/ATR substrate that is phosphorylated on *Ser/Thr* at SQ/TQ cluster domains (sATM/ATR^P) (47). The response induced by DNA replication inhibitors thus can be assessed by *detecting activation of ATR and phosphorylation of H2AX (expression of γ H2AX) concurrent with the lack of activation of ATM* (36). It should be noted, however that in certain instances the DNA replication stress may also induce activation of ATM and DNA-dependent protein kinase (DNA-PKcs) (48).

2.3. Analysis of DDR induced by DNA replication inhibitors

The protocol presented in this chapter is designed to assess intensity of γ H2AX and/or ATM-S1981^P and/or phosphorylated ATM/ATR substrate (sATM/ATR^P) immunofluorescence (IF) for evaluation of the degree of DNA damage response. The detection of γ H2AX, sATM/ATR^P or ATM-S1981^P is combined with differential staining of DNA to assess cellular DNA contents which reveals the cell cycle phase (*See* Note 1). The procedure is based either on *indirect* IF detection of these phosphorylated proteins using the primary Ab unlabeled and the secondary Ab conjugated with FITC or Alexa Fluor 488, or using *direct IF* with the fluorochrome-tagged primary Abs (*See* Note 2). DNA is counterstained with propidium iodide (PI) whose emission spectrum (red) is separated from the green color emission of FITC or Alexa Fluor 488. The cells are briefly fixed in methanol-free formaldehyde and then transferred into 70% ethanol in which they can be kept briefly (≥ 2 h) or stored at -20°C for weeks or longer. Ethanol treatment makes the plasma membrane permeable to the γ H2AX Ab; further permeabilization is achieved by including the detergent Triton X-100 into a solution used to incubate cells with the Ab. After incubation with the primary γ H2AX Ab the cells are incubated with FITC or Alexa Fluor 488-labeled secondary Ab and their DNA is counterstained PI in the presence of RNase A to remove RNA, which otherwise, similar as DNA, is also being stained with PI. Intensity of cellular green (FITC or Alexa Fluor 488) and red (PI) fluorescence is measured by flow cytometry.

It should be noted that H2AX undergoes constitutive phosphorylation in healthy cells, untreated by radiation or genotoxic agents. This constitutive expression of γ H2AX, which is more pronounced in S and G₂M than in G₁ cells, is considered to be in large part a reflection of oxidative DNA damage caused by the metabolically generated oxidants (49-50). It should also be noted that DNA fragmentation during apoptosis (51) leads to formation of large quantity of DSBs inducing high level of H2AX phosphorylation (52; *see* Figure 1). Strategies are presented to distinguish the DNA damage induced by topo inhibitors from the

¹On the bivariate distributions (scatterplots) subpopulations of cells in G₁, *versus* S *versus* G₂/M are distinguished based on differences in their DNA content (intensity of PI fluorescence; *See* Fig. 1). To assess the mean extent of DNA damage for cells at a particular phase of the cycle the mean values of γ H2AX, or ATM-S1981^P or sATM/ATR^P IF are calculated separately for G₁, S, and G₂/M cells distinguished based on differences in DNA content, by the computer-interactive "gating" analysis. The gating analysis should be carried out to obtain mean values of γ H2AX or ATM-S1981^P or sATM/ATR^P IF for G₁ (DNA Index; DI = 0.9–1.1), S (DI = 1.2–1.8) and G₂M (DI = 1.9–2.1) cell subpopulations.

²If primary Ab conjugated with fluorochrome (e.g. with FITC or Alexa Fluor 488) is being used replace the unlabelled Ab in step 5 with the conjugated one and move to step 10, omitting steps 6–9.

constitutive damage occurring in untreated cells (*See* note 3), or from the apoptosis-associated (AA) DSBs (*See* note 4).

2. Materials

2.1 Required reagents

- 1 Cells to be analyzed: $10^6 - 5 \times 10^6$ cells, untreated (control) and treated in culture with inhibitors of DNA replication (e.g. hydroxyurea, aphidicolin or thymidine at concentration generally used to inhibit DNA synthesis and synchronize cells at the entrance to S phase) suspended in 1 ml of tissue culture medium.
- 2 Methanol-free formaldehyde (Polysciences, Warrington, PA)
- 3 70 % ethanol
- 3 Phosphate-buffered saline (PBS)
- 4 Triton X-100 (Sigma Chemical Co., St. Louis, MO)
- 5 Bovine serum albumin (BSA; Sigma)
- 6 Abs: There are several commercially available phospho-specific Abs mono- as well as poly- clonal, unconjugated and FITC- or Alexa Fluor 488- conjugated, applicable to cytometry, that can be used to detect γ H2AX, ATM-S1981^P or ATM/ATR substrate [e.g. from BioLegend (San Diego, CA), Cell Signaling/ Santa Cruz Biotechnology (Danvers, MA), Molecular Probes/Invitrogen (Eugene, OR), Millipore/Upstate (Lake Placid, NY). The same companies also offer FITC- or Alexa 488- tagged secondary Abs to be used in conjunction with the non-conjugated primary Abs to these phosphorylated proteins.
- 8 Propidium iodide (PI; Molecular Probes/Invitrogen)
- 9 DNase-free RNase A (e.g. from Sigma)
- 10 12 × 75 mm polypropylene tubes
- 11 Centrifuge and rotor capable of 300g

³As mentioned in Introduction the low level of expression of γ H2AX or ATM-S1981^P IF seen in cells that have not been treated with exogenous inducers of DDR represents the constitutive ("background") H2AX phosphorylation and ATM activation. To quantify the γ H2AX or ATM-S1981^P IF induced by DNA replication inhibitors the constitutive component of γ H2AX IF or ATM-S1981^P has to be subtracted. Towards this end the means of γ H2AX or ATM-S1981^P IF of G₁, S and G₂/M-phase of the untreated cells are subtracted from the respective means of the inhibitor-treated cells, respectively (*See* Fig. 1.) After the subtraction the extent of *increase* in intensity of γ H2AX ($\Delta \gamma$ H2AX IF) or ATM-S1981^P (Δ ATM-S1981^P IF) over the untreated sample represents the *treatment-induced phosphorylation* of this protein, per each phase of the cell cycle. Alternatively, one can express the inhibitor-induced phosphorylation of H2AX or sATM/ATR as a percent (or multiplicity) of the increase of the mean IF value of the inhibitor-treated- to the mean of the untreated- cells, in the respective phases of the cell cycle. The irrelevant isotype control can be used to estimate the nonspecific Ab binding component, although its use may be unnecessary when one is interested only in the assessment of the inhibitor-induced increase (Δ) in IF.

⁴DNA undergoes extensive fragmentation during apoptosis (51) which leads to the appearance of a multitude of DSBs in apoptotic cells, triggering H2AX phosphorylation and ATM activation (52). It is often desirable, therefore, to distinguish between primary DSBs induced by DNA damaging agents such as DNA replication inhibitors *versus* DSBs generated during apoptosis. The following attributes of γ H2AX IF allow one to distinguish the cells with the inhibitor-induced H2AX phosphorylation from the cells that have phosphorylation of this histone triggered by apoptosis-associated (AA) DNA fragmentation: (i) The γ H2AX IF induced by replication inhibitors is seen rather early during the treatment (30 min - 2 h) whereas AA γ H2AX IF is seen later (>3h) (52); (b) The intensity AA γ H2AX IF is much higher than that of the inhibitor-induced γ H2AX IF, unless the cells are at late stage of apoptosis (52, *see* Figure 1); (c) The induction of AA γ H2AX IF is prevented by cell treatment with the caspase inhibitor z-VAD-FMK, which precludes activation of endonuclease responsible for DNA fragmentation. (52); (d) The AA H2AX phosphorylation occurs concurrently with activation of caspase-3. Multiparameter analysis [activated (cleaved) caspase-3 *versus* γ H2AX IF], thus, allows one to distinguish cells in which DSBs were caused by inducers of DNA damage (active caspase-3 is undetectable) from the cells that have H2AX phosphorylation and ATM activation additionally triggered in response to apoptotic DNA fragmentation (active caspase-3 is present). (e) ATM is activated in response to AA DNA fragmentation whereas is not activated during replication stress (36). These strategies are discussed in more detail elsewhere (46).

2.2. Reagents to be prepared

1. Methanol-free formaldehyde fixative: Prepare 1% (v/v) solution of methanol-free formaldehyde in PBS. This solution may be stored at 4°C for up to two weeks
2. BSA-T-PBS: Dissolve BSA in PBS to obtain 1 % (w/v) BSA solution. Add Triton X-100 to obtain 0.2% (v/v) of its concentration. This solution may be stored at 4 °C for up to two weeks.
3. PI stock solution: Dissolve PI in distilled water to obtain 1 mg/ml solution. This solution can be stored at 4°C in the dark (e.g. in the tube wrapped in aluminum foil) for several months.
4. PI staining solution: Dissolve RNase A (DNase-free) in PBS to obtain 0.1% (w/v; 100 mg/ml) solution. Add an appropriate aliquot of PI stock solution (e.g. 5 µl per 1 ml) to obtain its 5 µg/ml final concentration. Store the PI staining solution in the dark. This solution may be stored at 4°C for up to two weeks.

2.2. Instrumentation

Flow cytometers of different types, offered by several manufacturers, can be used to measure cell fluorescence following staining according to the protocol given below. The most common flow cytometers are from Coulter Corporation (Miami, FL), Becton Dickinson Immunocytometry Systems (San Jose, CA), PARTEC (Zurich, Switzerland), Accuri Cytometers Inc (Ann Arbor, MI), Millipore-Guava (Billerica, MA) or Applied Biosystems (Foster City, CA).

3. Methods

- 1 Centrifuge cells collected from tissue culture (suspended in culture medium) at 300g for 4 min at room temperature. Suspend cell pellet ($1-2 \times 10^6$ cells) in 0.5 ml of PBS.
- 2 With a Pasteur pipette transfer this cell suspension into polypropylene tube (*see* Note 5) containing 4.5 ml of ice-cold 1% methanol-free formaldehyde solution in PBS. Keep on ice for 15 min.
- 3 Centrifuge at 300g for 4 min at room temperature and suspend cell pellet in 4.5 ml of PBS. Centrifuge again as above and suspend cell pellet in 0.5 ml of PBS. With a Pasteur pipette, transfer the suspension to a tube containing 4.5 ml of ice-cold 70% ethanol. The cells should be maintained in 70% ethanol for 1 h but may be stored in 70 % ethanol at -20 °C for several weeks.
- 3 Centrifuge at 200g for 4 min at room temperature, remove the ethanol and suspend cell pellet in 2 ml of BSA-T-PBS solution.
- 4 Centrifuge at 300g for 4 min at room temperature and suspend the cells again in 2 ml of BSA-T-PBS. Keep at room temperature for 5 min.
- 5 Centrifuge at 300g for 4 min at room temperature and suspend the cells in 100 µl of BSA-T-PBS containing 1 µg of the primary γ H2AX Ab (*See* Notes 2 and 6).

⁵If the sample initially contains a small number of cells they may be lost during repeated centrifugations. Polypropylene or siliconized glass tubes are recommended to minimize cell loss. Since transferring cells from one tube to another causes electrostatic attachment of a large fraction of cells to the surface of each new tube, all steps of the procedure, including fixation, preferentially should be done in the same tube. Addition of 1% (w/v) BSA to rinsing solutions also decreases cell loss. When the sample contains very few cells, carrier cells (e.g., chick erythrocytes) may be included; they may be recognized during analysis based on differences in DNA content (intensity of PI fluorescence).

- 6 Cap the tubes to prevent drying and incubate them overnight at 4 °C (*See* Note 7).
- 7 Add 2 ml of BSA-T-PBS and centrifuge at 300g for 4 min at room temperature.
- 8 Centrifuge at 300g for 4 min at room temperature and suspend the cells in 2 ml of BSA-T-PBS.
- 9 Centrifuge at 300g for 4 min at room temperature and suspend the cells pellet in 100 μ l of BSA-T-PBS containing the appropriate (anti-mouse or anti-rabbit, depending on the source of the primary Ab) FITC- or Alexa Fluor 488- tagged secondary Ab (*See* Note 6).
- 10 Incubate for 1 h at room temperature, occasionally gently shaking. Add 5 ml of BSA-T-PBS and after 2 min centrifuge at 300g for 4 min at room temperature.
- 11 Suspend the cells in 1 ml of the PI staining solution. Incubate at room temperature for 30 min in the dark.
- 12 Set up and adjust the flow cytometer for excitation with light at blue wavelength (488-nm laser line or BG-12 excitation filter).
- 13 Measure intensity of green (530 ± 20 nm) and red (> 600 nm) fluorescence of the cells by flow cytometry. Record the data.

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References

1. Grdina, DJ.; Meistrich, ML.; Meyn, RE.; Johnson, TS.; White, RA. Cell synchrony techniques. A comparison of methods. In: Gray, JW.; Darzynkiewicz, Z., editors. *Techniques in Cell Cycle Analysis*. Clifton, New Jersey USA: Humana Press Inc; 1987. p. 367-402.
2. Davis PK, Ho A, Dowdy SF. Biological methods for cell-cycle synchronization of mammalian cells. *BioTechniques*. 2001; 30:1322–1331. [PubMed: 11414226]
3. Merrill GF. Cell synchronization. *Methods Cell Biol*. 1998; 57:229–249. [PubMed: 9648108]
4. Amon A. Synchronization procedures. *Methods Enzymol*. 2002; 351:457–467. [PubMed: 12073363]
5. Terasima T, Tolmach LJ. Growth and nucleic acid synthesis in synchronously dividing populations of HeLa cells. *Exp. Cell Res*. 1963; 30:344–362. [PubMed: 13980637]
6. Edward KL, Van Ert MN, Thornton M, Helmstetter CE. Cyclin mRNA stability does not vary during the cell cycle. *Cell Cycle*. 2004; 3:1057–1060. [PubMed: 15254409]
7. Thornton M, Edward KL, Helmstetter CE. Production of minimally disturbed synchronous cultures of hematopoietic cells. *BioTechniques*. 2002; 32:1098–10100. [PubMed: 12019783]
8. Huang X, Dai W, Darzynkiewicz Z. Enforced adhesion of hematopoietic cells to culture dish induces endomitosis and polyploidy. *Cell Cycle*. 2005; 4:801–805. [PubMed: 15908784]

⁶Quality of the primary and of secondary antibody is of utmost importance. The ability to detect γ H2AX, sATM/ATR^P or ATM-S1981^P is often lost during improper transport or storage conditions of the Ab. We occasionally observed that the Abs provided by the vendor were defective. Also of importance is use of the Abs at optimal concentration. It is recommended that with the first use of every new batch of the primary or secondary Ab to test them at serial dilution (e.g. within the range between 0.2 and 2.0 μ g/100 μ l) to find their optimal titer for detection of these phospho-proteins. The optimal titer is recognized as giving maximal signal to noise ratio, i.e. the maximal ratio of the mean IF intensity of the drug-treated to the untreated cells. Not always the titer recommended by the vendor is the optimal one.

⁷Alternatively, incubate for 1 h at 22–24 °C. The overnight incubation at 4 °C, however, appears to yield somewhat higher intensity of γ H2AX IF, sATM/ATR^P or ATM-S1981^P IF compared to 1h-incubation.

9. Jakóbsiak M, Bruno S, Skierski J, Darzynkiewicz Z. The cell cycle specific effects of lovastatin. *Proc. Natl. Acad. Sci. USA*. 1991; 88:3628–3613. [PubMed: 1673788]
10. Crissman HA, Gadbois DM, Tobey RA, Bradbury EM. Transformed mammalian cells are deficient in kinase-mediated progression through the G₁ phase of the cell cycle. *Proc. Natl. Acad. Sci. USA*. 1991; 88:7580–7585. [PubMed: 1652754]
11. Bruno S, Ardelt B, Skierski JS, Traganos F, Darzynkiewicz Z. Different effects of staurosporine, an inhibitor of protein kinases, on the cell cycle and chromatin structure of normal and leukemic lymphocytes. *Cancer Res*. 1992; 52:470–476. [PubMed: 1728418]
12. Bruno S, Traganos F, Darzynkiewicz Z. Cell cycle synchronizing properties of staurosporine. *Meth. Cell Sci*. 1996; 18:99–107.
13. Griffin MJ. Synchronization of some human cell strains by serum and calcium starvation. *In Vitro*. 1976; 12:393–400. [PubMed: 947840]
14. Tobey RA, Crissman HA. Use of flowmicrofluorimetry in detailed analysis of effects of chemical agents on cell cycle progression. *Cancer Res*. 1972; 32:2726–2731. [PubMed: 4118766]
15. Holley RW, Kiernan MA. “Contact inhibition” of cell division in 3T3 cells. *Proc. Natl. Acad. Sci. USA*. 1968; 60:300–305. [PubMed: 5241531]
16. Pardee AB, Keyomarsi K. Modification of cell proliferation with inhibitors. *Curr. Opin. Cell Biol*. 1992; 4:186–191. [PubMed: 1376129]
17. Mitchell BF, Tupper JT. Synchronization of mouse 3T3 and SV40 3T3 cell by way of centrifugal elutriation. *Exp. Cell Res*. 1977; 106:351–355. [PubMed: 193701]
18. Cymerman U, Beer JB. Some problems in using density gradient centrifugation for synchronization of L5178Y-S cells. *Neoplasma*. 1980; 27:429–436. [PubMed: 7453858]
19. Juan G, Hernando E, Cordon-Cardo C. Separation of live cells in different phases of the cell cycle for gene expression analysis. *Cytometry*. 2002; 49:170–175. [PubMed: 12454980]
20. Zhao H, Traganos F, Dobrucki J, Wlodkowic D, Darzynkiewicz Z. Induction of DNA damage response by the supravital probes of nucleic acids. *Cytometry A*. 2009; 75A:510–519. [PubMed: 19373929]
21. Zhang X, Chen J, Davis B, Kiechle F. Hoechst 33342 induces apoptosis in HL-60 cells and inhibits topoisomerase I *in vivo*. *Arch. Pathol. Lab. Med*. 1999; 123:921–927. [PubMed: 10506445]
22. Mohorko N, Kregar-Velikonja N, Repovs G, Gorenssek M, Bresjanac M. An *in vitro* study of Hoechst 33342 redistribution and its effects on cell viability. *Hum. Exp. Toxicol*. 2005; 24:573–580. [PubMed: 16323574]
23. Samake S, Smith LC. Synchronization of cell division in eight-cell bovine embryos produced *in vitro*: effects of nocodazole. *Mol. Reprod. Dev*. 1996; 44:486–492. [PubMed: 8844691]
24. Harper JV. Synchronization of cell populations in G₁/S and G₂/M phases of the cell cycle. *Methods Mol. Biol*. 2005; 296:157–165. [PubMed: 15576930]
25. Darzynkiewicz Z, Crissman H, Traganos F, Stainkamp J. Cell heterogeneity during the cell cycle. *J. Cell Physiol*. 1982; 112:465–474. [PubMed: 6184378]
26. Urbani L, Sherwood SW, Schimke RT. Dissociation of nuclear and cytoplasmic cell cycle progression by drugs employed in cell synchronization. *Exp. Cell Res*. 1995; 219:159–168. [PubMed: 7628532]
27. Chou LF, Chou WG. Dna-end binding activity of Ku in synchronized cells. *Cell Biol. Int*. 1999; 23:663–670. [PubMed: 10736189]
28. Piotrowska K, Modlinski JA, Korwin-Kossakowski M, Karasiewicz J. Effects of preactivation of ooplasts or synchronization of blastomere nuclei in G₁ on preimplantation development of rabbit serial nuclear transfer embryos. *Biol. Reprod*. 2000; 63:677–682. [PubMed: 10952907]
29. Tobey RA, Oishi N, Crissman HA. Cell cycle synchronization: Reversible induction of G₂ synchrony in cultured rodent and human diploid fibroblasts. *Proc. Natl. Acad. Sci. USA*. 1990; 87:5104–5109. [PubMed: 1695009]
30. Vogel W, Schempp W, Sigwarth I. Comparison of thymidine, fluorodeoxyuridine, hydroxyurea, and methotrexate blocking at the G₁/S phase transition of the cell cycle, studied by replication patterns. *Hum. Genet*. 1978; 45:193–198. [PubMed: 153886]

31. Fox MH, Read RA, Bedford JS. Comparison of synchronized Chinese hamster ovary cells obtained by mitotic shake-off, hydroxyurea, aphidicolin, or methotrexate. *Cytometry*. 1987; 8:315–320. [PubMed: 3109858]
32. Matherly LH, Schuetz JD, Westin E, Goldman ID. A method for the synchronization of cultured cells with aphidicolin: application to the large-scale synchronization of L1210 cells and the study of the cell cycle regulation of thymidylate synthase and dihydrofolate reductase. *Anal. Biochem.* 1989; 182:338–345. [PubMed: 2514611]
33. Kues WA, Anger M, Carnawath JW, Paul D, Motlik J, Niemann H. Cell cycle synchronization of porcine fetal fibroblasts: effects of serum deprivation and reversible cell cycle inhibitors. *Biol. Reprod.* 2000; 62:412–419. [PubMed: 10642581]
34. Cohen LS, Studzinski GP. Correlation between cell enlargement and nucleic acid and protein content of HeLa cells in unbalanced growth produced by inhibitors of DNA synthesis. *J. Cell Physiol.* 1967; 69:331–339. [PubMed: 4230858]
35. Gong J, Traganos F, Darzynkiewicz Z. Growth imbalance and altered expression of cyclins B1, A, E and D3 in MOLT-4 cells synchronized in the cell cycle by inhibitors of DNA replication. *Cell Growth & Differentiation*. 1995; 6:1485–1492. [PubMed: 8562487]
36. Kurose A, Tanaka T, Huang X, Traganos F, Dai W, Darzynkiewicz Z. Effects of hydroxyurea and aphidicolin on phosphorylation of ATM on Ser 1981 and histone H2AX on Ser 139 in relation to cell cycle phase and induction of apoptosis. *Cytometry A*. 2006; 69A:212–221. [PubMed: 16528719]
37. Kurose A, Tanaka T, Huang X, Traganos F, Darzynkiewicz Z. Synchronization in the cell cycle by inhibitors of DNA replication induces histone H2AX phosphorylation, an indication of DNA damage. *Cell Prolif.* 2006; 39:231–240. [PubMed: 16672000]
38. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine139. *J. Biol. Chem.* 1998; 273:5858–5868. [PubMed: 9488723]
39. Sedelnikova OA, Rogakou EP, Panuytin IG, Bonner W. Quantitative detection of ¹²⁵IUdr-induced DNA double-strand breaks with γ -H2AX antibody. *Radiation Res.* 2002; 158:486–494. [PubMed: 12236816]
40. Mah L-J, El-Osta A, Karagiannis TC. γ H2AX: a sensitive molecular marker of DNA damage and repair. *Leukemia*. 2010 Epub Feb 14.
41. Lavin MF, Kozlov S. ATM activation and DNA damage response. *Cell Cycle*. 2007; 6:931–942. [PubMed: 17457059]
42. Zhao H, Traganos F, Darzynkiewicz Z. Kinetics of the UV-induced DNA damage response in relation to cell cycle phase. Correlation with DNA replication. *Cytometry A*. 2010; 74A (Epub. Jan 25).
43. Tanaka T, Huang X, Halicka HD, Zhao H, Traganos F, Albino AP, Dai W, Darzynkiewicz Z. Cytometry of ATM activation and histone H2AX phosphorylation to estimate extent of DNA damage induced by exogenous agents. *Cytometry A*. 2007; 71A:648–661. [PubMed: 17622968]
44. MacPhail SH, Banath JP, Yu TY, Chu EH, Lambur H, Olive PL. Expression of phosphorylated histone H2AX in cultured cell lines following exposure to X-rays. *Int. J. Radiat. Biol.* 2003; 79:351–358. [PubMed: 12943243]
45. Zhao H, Traganos F, Darzynkiewicz Z. Kinetics of histone H2AX phosphorylation and Chk2 activation in A549 cells treated with topotecan and mitoxantrone in relation to the cell cycle phase. *Cytometry A*. 2008; 73A:480–489. [PubMed: 18459160]
46. Kurose A, Tanaka T, Huang X, Halicka HD, Traganos F, Dai W, Darzynkiewicz Z. Assessment of ATM phosphorylation on Ser-1981 induced by DNA topoisomerase I and II inhibitors in relation to Ser-139-histone H2AX phosphorylation, cell cycle phase and apoptosis. *Cytometry A*. 2005; 68A:1–9. [PubMed: 16184611]
47. Traven A, Heierhorst J. SQ/TQ cluster domains: concentrated ATM/ATR kinase phosphorylation site regions in DNA-damage-response proteins. *BioEssays*. 2005; 27:397–407. [PubMed: 15770685]

48. Yajima H, Lee KJ, Zhang S, Kobayashi J, Chen BP. DNA double-strand break formation upon UV-induced replication stress activates ATM and DNA-PKcs kinases. *J. Mol. Biol.* 2009; 385:800–810. [PubMed: 19071136]
49. Tanaka T, Halicka HD, Huang X, Traganos F, Darzynkiewicz Z. Constitutive histone H2AX phosphorylation and ATM activation, the reporters of DNA damage by endogenous oxidants. *Cell Cycle.* 2006; 5:1940–1945. [PubMed: 16940754]
50. Zhao H, Tanaka T, Halicka HD, Traganos F, Zarebski M, Dobrucki J, Darzynkiewicz Z. Cytometric assessment of DNA damage by exogenous and endogenous oxidants reports the aging-related processes. *Cytometry A.* 2007; 71A:905–914. [PubMed: 17879239]
51. Kajstura M, Halicka HD, Pryjma J, Darzynkiewicz Z. Discontinuous fragmentation of nuclear DNA during apoptosis revealed by discrete “sub-G₁” peaks on DNA content histograms. *Cytometry A.* 2007; 71A:125–131. [PubMed: 17252584]
52. Tanaka T, Kurose A, Huang X, Dai W, Darzynkiewicz Z. ATM kinase activation and histone H2AX phosphorylation as indicators of DNA damage by DNA topoisomerase I inhibitor topotecan and during apoptosis. *Cell Prolif.* 2006; 39:49–60. [PubMed: 16426422]

Fig. 1. Detection of histone H2AX phosphorylation and ATM activation in HL-60 cells treated with DNA polymerase alpha inhibitor aphidicolin (Aph)

The cells were untreated (Ctrl) or treated in culture with 1 or 4 μ M Aph and expression of γ H2AX and ATM-S1981^P was measured as described in the protocol (indirect immunofluorescence). The bivariate distributions (scatterplots) of γ H2AX or ATM-S1981^P immunofluorescence (IF) vs DNA content allow one to identify subpopulations of cells in G₁, S and G₂M phases of the cell cycle as shown in left panels. Increased expression of γ H2AX is primarily seen in cells at the interphase of G₁ and S (marked by rectangular dashed outlines) and also in S and G₂M in cells after treatment with Aph. No increase in expression of ATM-S1981^P is apparent. Apoptotic cells (Ap) are detected as having

markedly elevated expression of both γ H2AX and ATM-S1981^P after treatment with 4 μ M Aph for 4 h. Note that the γ H2AX and ATM-S1981^P coordinates are exponential while DNA content scale is linear.