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Reversing the Effect of Formalin on the Binding of Propidium Iodide to DNA[1,2]

W. Roy Overton[3] and J. Philip McCoy, Jr.
Department of Pediatrics, Division of Hematology/Oncology, Cooper Hospital/UMC, Camden, New Jersey 08103

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Formalin, an excellent preservative of cellular morphology, is a commonly used fixative for tissue specimens in hospital pathology laboratories. This preserved material is a potential source of tissue for diagnostic and retrospective research studies on DNA using flow cytometry. Unfortunately, formalin interferes with the binding of propidium iodide (PI) and other fluorescent dyes to DNA, thus altering the measurement of DNA content by flow cytometry or image analysis. This interference has been attributed to the cross-linking of histones by formalin. Since formalin alters the measurement of DNA content in formalin-fixed and formalin-fixed, paraffin-embedded tissues, this study was designed to explore the use of various physicochemical methods to reverse the effect of the formalin on the binding of PI to DNA. This study demonstrates that resuspending formalin-fixed cells in PBS and heating them at 75°C for at least 1 h prior to staining with PI restores the staining of the DNA to approximately the same fluorescence intensity as that of fresh tissue.

Key terms: Formalin fixation, propidium iodide, DNA, flow cytometry

Cellular morphology can be preserved by fixing cells in formalin (14). Therefore, surgical and autopsy tissue specimens are commonly fixed in 10% buffered formalin and then embedded in paraffin for morphological examination and long-term storage. This practice has resulted in the accumulation in many pathology departments of archived tissue that could be of use in retrospective studies of neoplasms, such as correlating patient prognosis with DNA-ploidy or cell cycle. Hedley et al. (11) developed a method for analyzing the DNA content of formalin-fixed, paraffin-embedded (FFPE) cells by flow cytometry. This method dewaxes and rehydrates the tissue and prepares and stains single cell suspensions of the tissue. This has been the method of choice for DNA analysis of FFPE tissues for the past 10 years. However, this methodology has been widely criticized for its inaccuracies in DNA-ploidy determination (5,7,8,13,15,16). These criticisms stem from the fact that fluorochromes that bind to DNA do not stain FFPE tissue with the same intensity as they do fresh tissue. This difference is reportedly due to the cross-linking of DNA and histones in formalin-fixed DNA (3,6). The formaldehyde in formalin causes the formation of methylene bridges between amino groups in the DNA and amino groups in the histones, and this cross-linking interferes with the dye binding to the DNA, as was discussed by Hedley et al. (11). This interference with dye intercalation into the DNA results in a reduced nuclear fluorescence and, thus, a shift in the apparent G_0/G_1 peak. Unfortunately, this effect is not consistent from one type of tissue to another (10) or even within the same kind of tissue (1). This inter- and intratissue variability of the effect of formalin makes DNA determinations on FFPE tissue even less reliable. Since the fluorochrome binding to formalin-fixed DNA is variable from tissue to tissue, it is not possible to use fresh or even fixed DNA-diploid cells as internal standards when making DNA-ploidy determinations of formalin-fixed or FFPE tissues.

Numerous efforts have been made to improve upon the Hedley et al. method by various enzyme treatments (17–19,21), but these modifications have resulted in only minor improvements, if any. However, these im-

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[3]Address reprint requests to W. Roy Overton, Ph.D., Flow Cytometry Laboratory, Division of Pediatric Hem/Onc, Three Cooper Plaza, Suite 410, Camden, NJ 08103.

Table 1
Effects of Formalin Fixation on PI Binding to DNA[a]

Cells	Fixation	N	G_0/G_1 fluor. intensity (% of fresh)	G_0/G_1 CV
WBC	None	18	100.0	2.42 ± 0.64
	None[b]	14	101.0 ± 2.6	2.71 ± 1.35
	Formalin	17	45.4 ± 5.6	9.59 ± 3.03
BMA	None	2	100.0	2.60 ± 0.57
	Formalin	2	43.2 ± 2.6	12.30 ± 0.99
T24	None	5	100.0	2.44 ± 0.38
	Formalin	5	62.5 ± 9.3	11.32 ± 2.61
A673	None	2	100.0	2.85 ± 0.07
	Formalin	2	62.4 ± 4.3	12.90 ± 1.41
SK-NEP-1	None	1	100.0	1.98
	Formalin	1	59.8	7.52

[a]Data are given as means ± standard deviations. CVs were calculated for full peaks. Percentage of fresh is calculated by dividing the mean channel of the test sample by the mean channel of the fresh (not fixed) sample run at the beginning of each experiment.
[b]Fresh WBC rerun at the end of experiments to check for possible instrument drift.

provements have centered around reducing the coefficient of variation of the G_0/G_1 peaks or decreasing apparent debris. No attempt has been made to restore dye binding to that of fresh tissue. Thus DNA analysis of FFPE tissue continues to be problematic.

It has been observed that the cross-linking of formaldehyde-fixed DNA histones is reversible by dialyzing formaldehyde-fixed chromatin in either a buffered salt solution at 37°C for 48 h (4) or in a 2-mercaptoethanol and guanidine hydrochloride solution at 100°C for 30 min (12). It has also been found that formaldehyde-induced changes in ultraviolet (UV) absorbance of polynucleotides are reversible by heating the polynucleotides in a phosphate solution at 75°C for several minutes (9). This reversibility of formaldehyde-induced cross-linking of histones in nucleosomes suggests that it may be possible to reverse the formaldehyde-induced changes in the binding of PI to DNA in intact nuclei. We report here several physicochemical methods for reversing the effect of formalin on the binding of PI to DNA.

MATERIALS AND METHODS

Tissue Preparation

Fresh normal leukocytes (WBCs) were obtained by ammonium chloride lysis of peripheral whole blood from healthy donors. Leukocytes from bone marrow were obtained by ammonium chloride lysis of a bone marrow aspirate (BMA). Cell lines were provided by Drs. Lydia McMorrow and Grzegorz Gorski. The cell lines included a bladder epithelial cell line (T24), a rhabdomyosarcoma cell line (A673), and a Wilms tumor line (SK-NEP-1). Cell lines were grown in high-glucose D-MEM medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and each ml of medium contained 100 U of penicillin G sodium, 100 U streptomycin sulfate, and 0.25 μg Fungizone.

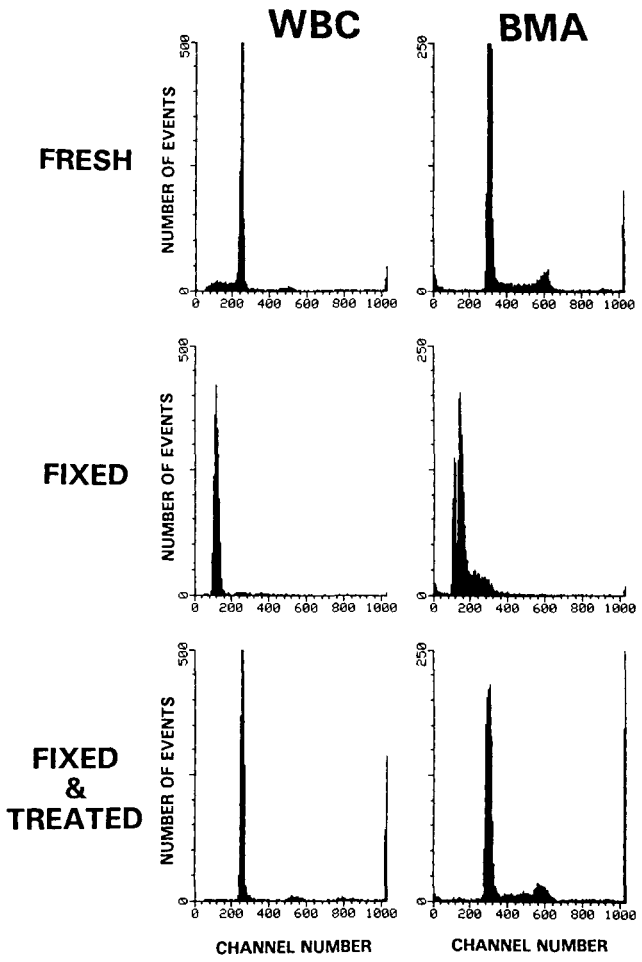

FIG. 1. Propidium iodide fluorescence of fresh and formalin-fixed WBC and BMA: The PI fluorescence is shown as ungated histograms for peripheral blood leukocytes (WBC) and for leukocytes from bone marrow aspirates (BMA). The histograms at the top of the figure are from fresh (not fixed) cells. The center two histograms are from formalin-fixed cells, and the bottom two histograms are from formalin-fixed cells incubated in PBS for 1 h at 75°C.

Fixation of Cell Suspensions

The 10% formalin solution, buffered in monobasic sodium phosphate (4 g/liter) and dibasic sodium phosphate (6.5 g/liter), was obtained from Curtin Matheson Scientific, Inc. (Houston, TX; Cat. No. 245-684). Aliquots of approximately 1×10^6 cells of each type studied were fixed by centrifuging (400*g*) the cells, removing the supernatant, and resuspending the cell pellet in 4 ml of the 10% buffered formalin described above by vortexing. The cells were incubated in the 10% formalin at room temperature for 1–24 h.

Treatment of Cell Suspensions

The fresh and fixed cells were washed twice in 4 ml Dulbecco's PBS (Gibco) and centrifuged (400*g*), and the

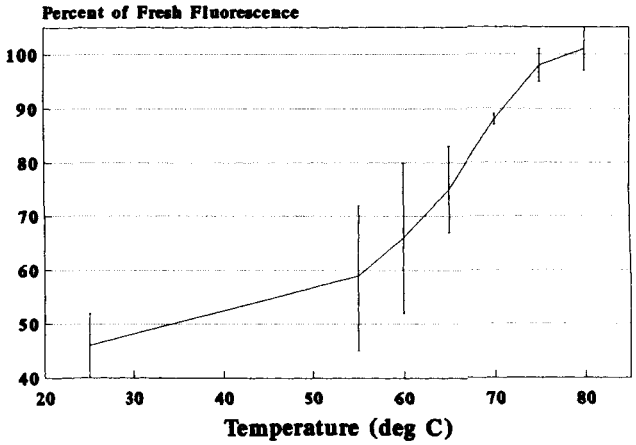

FIG. 2. Propidium iodide fluorescence of formalin-fixed WBCs treated with heat: The PI fluorescence of formalin-fixed WBCs incubated in PBS at various temperatures for 1 h is shown compared to the PI fluorescence of fresh cells. These data are presented as a percentage of the fluorescence intensity of fresh cells, analyzed within the experiment, and are given as mean ± standard deviation. The results from four experiments are shown for each temperature (N = 4) except for 25°C and 75°C, for which the results of nine additional experiments were included (N = 13).

supernatant was removed. Each sample, containing approximately 1×10^6 of the fixed cells, was then resuspended in 4 ml of one of the following solutions: 1) deionized water, 2) Dulbecco's PBS, 3) 10% methanol in water, 4) 1-12 N HCl, 5) 1-12 N H_2SO_4, 6) 1-12 N NaOH, and 7) 0.5 M 2-mercaptoethanol (2-ME) with 2 M guanidine hydrochloride. The cells were vortexed and then incubated either on ice (4°C), at room temperature (25°C), or in a water bath at temperatures ranging from 37°C to 80°C for various incubation periods up to 4 h. The cells were then washed twice in 4 ml Dulbecco's PBS.

DNA Staining

The cells were centrifuged (400*g*), and the supernatant was removed. Each aliquot of cells was then stained by resuspending the pellet in 1 ml PI solution that contained 50 μg PI, 10 mM Trizma base, 10 mM NaCl, 0.7 U RNAse, and 1 μl NP-40 and then vortexing. The cells were incubated in the PI solution at 4°C for at least 1 h before flow cytometric analysis was performed.

Flow Cytometry

Flow cytometry was performed on a Becton-Dickinson FACScan equipped with LYSIS II software. Red-orange fluorescence (FL2) passing through a 585 nm bandpass filter was used to trigger signal processing, and the area and width of the linearly amplified FL2 pulses were collected in list mode for 10,000 events. For each sample, an ungated histogram was generated showing the linear fluorescence intensity for all events collected. This ungated histogram shows not only the single nuclei but also the debris and doublets present in each sample. Cell cycle analysis was performed using ModFit (Verity Software House, Topsham, ME).

The mean channel of the G_0/G_1 peak for each sample of fixed cells (untreated and treated) was divided by the mean channel of the G_0/G_1 peak of the fresh cells from the same donor sample or tissue culture sample. Multiplying this calculation by 100 yields a percentage of fresh cell fluorescence intensity.

RESULTS

The PI staining of each type of cell was *decreased* by formalin fixation. In Table 1, the fluorescence intensity of the fixed cells is compared to that of the fresh cells. Table 1 also shows the increase in coefficient of variation of the G_0/G_1 peak after the cells are fixed in formalin. The leukocytes from the peripheral blood and from the bone marrow had a greater decrease in fluorescence intensity as a result of the formalin fixation than did the cultured cell lines. This difference between the WBC and T24 is significant ($P < 0.02$; unpaired t test) and is consistent with previous findings that various cell types are affected differently by formalin fixation (10). Shifts in fluorescence due to instrument drift were detected by reanalysis of the fresh WBC sample at the end of the experiment, as shown in Table 1, and were found to be nominal.

As can be seen in the ungated histograms in Figure 1, the leukocytes from both peripheral blood and from bone marrow decrease in PI fluorescence intensity, but this decrease can be reversed by heating the cells in PBS at 75°C for 1 h. The temperature dependence of the PBS treatment is shown in Figure 2. Similarly, examples of the effect of formalin and the reversal of this effect by the 1 h incubation in PBS at 75°C are shown for the three cell lines in Figure 3.

Of the treatments tested in this study, incubating the cells at 75°C in PBS for 1–2 h was the most effective in restoring the PI fluorescence to approximately that of the fresh cells. Heating the cells in H_2O or 10% methanol in H_2O gave results similar to those of heating in PBS.

Low concentrations of hydrochloric or sulfuric acid (≤4 N) at room temperature slightly increased the fluorescence intensity of formalin-fixed cells, but higher concentrations of acid or warming the acid to 37°C reduced the fluorescence intensity to less that of the untreated, fixed cells. The intensity of the PI staining was inversely correlated with the acid concentration and incubation time when the acid treatment was done at 37°C. Fluorescence could not be analyzed due to the loss of the cells when the cells were incubated for 2 h or more in 8 N HCl or for 4 h in 2 N or stronger HCl.

Treatment with NaOH increased PI staining of formalin-fixed cells but to an intensity less than that of fresh cells. This increase was maximized when the cells were warmed to 37°C for 1 h in 6 N NaOH (Table 2). Heating the cells to 55°C or greater with NaOH (≥1 N) resulted in loss of cells.

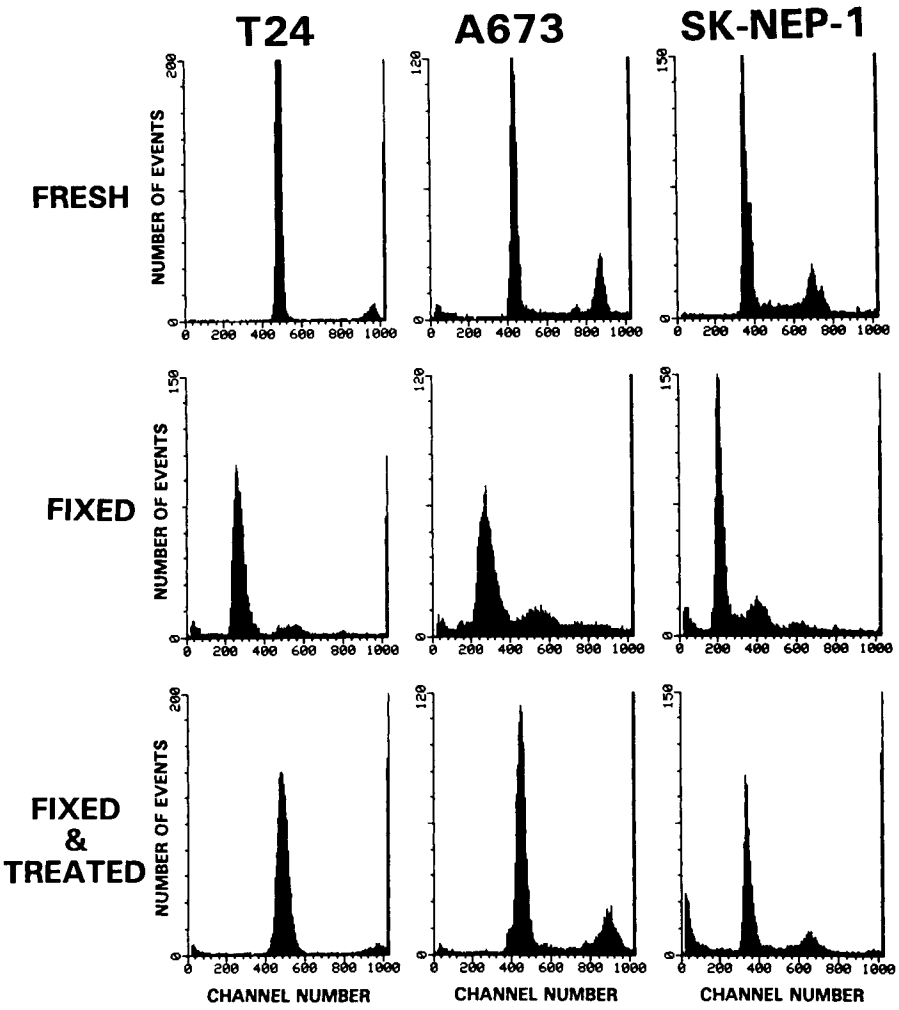

FIG. 3. Propidium iodide fluorescence of formalin-fixed cell lines: The PI fluorescence is shown as ungated histograms for cultured cell lines T24, A673, and SK-NEP-1. The histograms at the top are from fresh (not fixed) cells. The center three histograms are from formalin-fixed cells, and the bottom three histograms are from formalin-fixed cells incubated in PBS for 1 h at 75°C.

Formalin-fixed WBCs and BMA were incubated in 2-ME at 25°C, 37°C, and 55°C for up to 4 h. Incubation of the fixed cells in 2-ME for 4 h at 55°C was found to restore the PI fluorescence to approximately that of fresh cells (Table 2). Incubating the cells in 2-ME at lower temperatures or for a shorter time was not as effective at restoring the PI fluorescence.

Fresh WBC were added to fresh T24 cells prior to formalin fixation in order to perform DNA-ploidy analysis and cell cycle analysis on the fixed T24 cells using the WBC as an internal DNA-diploid control. As is shown in Table 3, formalin fixation alters the DNA index and the cell cycle analysis of the T24 cells. The incubation of the formalin-fixed T24 and WBC cell mixture restores both the DNA index and the cell cycle statistics to approximately those of the fresh cells (Table 3).

DISCUSSION

All measurements made by flow cytometry are relative. Each measurement must be related to another in order to make the data meaningful. This is true for light scatter as well as fluorescence. Therefore, to make a quantitative measurement by flow cytometry, it is necessary to relate the measurement to a "known" or standard. In measuring DNA content of cells, the fluorescence intensity of DNA dyes directly correlates with the amount of DNA in the cell. However, measurement of DNA is affected by many parameters, including laser power, fluorescence efficiency of the dye, salt concentration, instrument sensitivity, and signal amplification. Therefore, DNA measurement is not quantitative unless the fluorescence of the unknown cells is directly compared to that of cells with known

Table 2
Reversal of Formalin Fixation Effects on PI Binding to DNA[a]

Cells	Postfixation treatment	N	G_0/G_1 Fluor. intensity (% of fresh)	G_0/G_1 CV
WBC	PBS @ 75°C	16	98.3 ± 4.1	3.80 ± 0.91
	NaOH @ 37°C	8	87.6 ± 10.2	4.00 ± 1.07
	2-ME @ 55°C	1	103.2	3.81
BMA	PBS @ 75°C	2	95.4 ± 2.4	3.95 ± 0.07
	NaOH @ 37°C	1	86.8	3.00
	2-ME @ 55°C	1	102.6	5.54
T24	PBS @ 75°C	4	105.2 ± 4.1	4.25 ± 0.65
	NaOH @ 37°C	3	98.5 ± 14.0	3.83 ± 1.36
A673	PBS @ 75°C	1	103.5	4.60
	NaOH @ 37°C	2	94.4 ± 9.7	6.00 ± 0.99
SK-NEP-1	PBS @ 75°C	1	98.3	5.44

[a]Data given as means ± standard deviations. CVs were calculated for full peaks. Percentage of fresh is calculated by dividing the mean channel of the test sample by the mean channel of the fresh (not fixed) sample run at the beginning of each experiment. Data for peripheral blood leukocytes (WBC); bone marrow aspirates (BMA); and cell lines T24, A673, and SK-NEP-1 are given for formalin-fixed cells incubated either in PBS at 75°C for 1–2 h, 6 N NaOH at 37°C for 1 h, or 2-mercaptoethanol at 55°C for 4 h.

DNA content, stained, and analyzed under identical conditions. DNA-ploidy determinations cannot be made without this quantitative comparison.

Formalin fixation of cells has a profound inhibitory effect on the intercalation of propidium iodide into DNA. The degree to which formalin inhibits PI binding is variable, but formalin can reduce the fluorescence of the PI-stained DNA by about one-half in some cell types. This reduction in PI staining of DNA seriously limits the value of measuring the PI fluorescence for DNA-ploidy determination. Because there is no appropriate standard that can be used with formalin-fixed tissue, it is not possible to determine correctly the DNA index of cells that have been fixed in formalin. However, it is common practice to determine DNA-ploidy in formalin-fixed cells by observing the presence of multiple peaks, which indicates that a DNA-aneuploid population is present (2,7,11,16). Since the formalin has shifted the peaks, it is not possible to determine which, if any, is DNA-diploid and whether the DNA-aneuploid population is DNA-hyperdiploid or DNA-hypodiploid. Conversely, a single G_0/G_1 peak is usually interpreted as DNA-diploid regardless of the fluorescence intensity and true DNA content. Classifying a single G_0/G_1 peak as "DNA-diploid" without having any DNA standard to relate it to is certainly not an ideal approach to DNA-ploidy determination.

The detection of DNA-aneuploid populations can be of some value regardless of the DNA content of the DNA-aneuploid population, but, recently, studies have shown that DNA-aneuploid populations may be erroneously detected or overlooked in formalin-fixed cells (5,7,8,13,15,16,18,19). It is essential, then, that methods be developed to reverse these effects of formalin on the determination of DNA content so that correct DNA-ploidy determinations can be made.

Several of the methods presented here are capable of reversing formalin's inhibitory effect on PI binding to DNA. The simplest and most effective method found in this study is the heating of the cells to 75°C for at least 1 h in PBS. This results in the restoration of the PI staining to approximately that of fresh cells regardless of the type of cell. This finding is consistent with that of Haselkorn and Doty (9), who found that the UV absorbance of polynucleotides is shifted by formaldehyde fixation and that the UV absorbance is restored to that of fresh by heating the fixed polynucleotides in phosphate solution at 75°C.

The mechanism for the heat-induced reversal of the effect of formalin has not been elucidated, but it may be a result of either a breaking of the methylene bridges that create the cross-linking of the histones and DNA or a denaturation of the DNA resulting in the exposure of non-cross-linked DNA. Either mechanism would result in an increased binding of PI to DNA. We have found that cells that are fixed, heated in PBS, and then

Table 3
Effects of Formalin Fixation on Cell Cycle Analysis[a]

			Diploid (WBC)				Aneuploid (T24)				
			G_0/G_1		S	G_2/M	G_0/G_1		S	G_2/M	
Fixation	Postfixation treatment	N	CV	%	%	%	CV	%	%	%	DI
None	None	3	1.96	89.0	9.2	1.7	2.14	96.3	3.1	0.7	1.81
			±0.42	±2.8	±1.6	±1.7	±0.08	±1.4	±1.6	±0.2	±0.04
Formalin	None	3	8.70	62.9	21.3	15.8	9.9	52.6	20.2	10.1	2.49
			±0.28	±36.08	±28.0	±12.0	±1.67	±42.2	±8.1	±9.4	±0.56
	PBS @ 75°C	3	3.79	96.3	3.4	0.2	3.90	94.7	4.8	0.5	1.94
			±1.10	±1.8	±2.0	±0.4	±0.79	±6.5	±5.8	±0.8	±0.08
	NaOH @ 37°C	2	5.56	83.3	3.3	13.5	7.14	80.7	14.6	4.8	2.08
			±2.74	±17.3	±0.1	±17.0	±2.34	±17.5	±12.7	±4.7	±0.35

[a]Data generated from samples of mixed WBC and T24 cells. Cells were mixed prior to fixation and staining. Data are given as means ± standard deviations. CVs were calculated for full peaks. Postfixation treatments were either incubation in PBS at 75°C for 1 h or incubation in 6 N NaOH at 37°C for 1 h. DNA index (DI) was calculated using the WBC as an internal diploid control.

fixed again will have reduced PI binding similar to that of cells fixed only once. A second heat treatment will again reverse the inhibition of the PI binding. The fact that PI staining can be repeatedly blocked by formalin and unblocked by heat treatment suggests that these effects may be due, at least in part, to the formation and subsequent removal of methylene bridges. Some denaturation of the DNA may also be occurring when the fixed cells are heated above 75°C, since this was found to increase the PI fluorescence to a slightly higher level than that of fresh cells. Traganos and his coworkers (20) have studied heat-denaturation of formalin-fixed DNA and have found that little or no denaturation occurs below 85°C in PBS. It seems likely, then, that the primary mechanism of the 75°C heat treatment is the removal of the methylene bridges that cross-link the histones and DNA.

Additional studies are ongoing to determine whether any of these methods can be used to restore PI fluorescence in formalin-fixed, paraffin-embedded tissues to the same intensity as in fresh tissue. Preliminary data suggest that heating the FFPE cells to 75°C in PBS for at least 1 h may restore the binding of PI to the DNA of FFPE tissue, as it does in formalin-fixed cells. However, extensive studies will be necessary to determine whether any such method could produce reliable DNA-ploidy determinations for tissue that has been formalin fixed and paraffin embedded.

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