Automatic analysis of slides processed in the Comet assay

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In recent years the Comet assay (or single cell gel electrophoresis assay) has been established as a rapid and sensitive method for the detection of DNA damage. For early genotoxicity screening of new chemical entities in industrial toxicology, the Comet assay is more and more used for assessment of the DNA damaging potential of a test compound. In order to increase compound screening throughput, we have established an image analysis system for fully automated measurement of microscope slides processed in the Comet assay. For the comparative investigation various cell types, such as V79 Chinese hamster cells, mouse lymphoma cells and human leukocytes, were treated with several test compounds. Using tail moment as the quantitative parameter for comet formation, we show a very high correlation between our automatic image analysis system and a commercially available, interactive system (Comet Assay II of Perceptive Instruments). The possibility of analyzing 50 samples within 1 day and the high reproducibility of results make automated image processing a powerful tool for automatic analysis of slides processed in the Comet assay.

Introduction

The alkaline Comet assay is a well-established genotoxicity test that has been used to detect a broad spectrum of DNA damage with high sensitivity (Fairbairn *et al.*, 1995; Anderson *et al.*, 1998; Rojas *et al.*, 1999). Guidelines for conduct of the assay have recently been published (Tice *et al.*, 2000). In this microgel electrophoresis test cells embedded in an agarose gel on a microscope slide are lysed, alkali denatured and electrophoresed. After fluorescence staining with propidium iodide, cells with increased DNA damage show increased comet-shaped migration of chromosomal DNA towards the anode. In the alkaline version of this test DNA strand breaks and alkali-labile sites are detectable and the extent of DNA migration indicates the amount of DNA damage in the cell (Singh *et al.*, 1988).

Image analysis for quantification of comet intensity was first reported by Olive (Olive, 1989). While several interactive image analysis systems based on the PC have been established in the meantime, automatic comet analysis has only recently been developed (Böcker *et al.*, 1999). To facilitate fast screening of large numbers of new compounds in industrial toxicology, complete automation of Comet assay analysis would be a big advantage.

Originally the extent of DNA damage was defined as the head-to-tail ratio by measuring the diameter of the head at a certain staining intensity and dividing by the distance between the head and the end of the comet tail (Olive, 1989). Various other parameters for comet quantification have since been used (for a review see Ashby *et al.*, 1995; Böcker *et al.*, 1997a, 1999).

We decided to use the tail moment as described by Olive *et al.* (1990) for the following reasons:
- easy computation through a simple, closed formula (for details see Materials and methods, Image analysis process);
- robustness with respect to degrading fluorescence illumination;
- independence of comet shape;
- well-established parameters which are also used by the interactive comet analysis system.

For fast, sensitive and unbiased analysis of slides we developed an image analysis system with the goal of minimizing user interaction. Except for slide loading and specification of the sample identifier and a starting field at the beginning, no manual steps should be necessary. The resulting system allows fully automatic analysis of 50 comets, which were automatically selected for comet analysis, in <10 min. Through placing two different samples on one slide and by using an electronically driven microscope with a stage capacity of eight slides, up to 16 samples can be analyzed in one run without further user interaction.

This report focuses on the technical aspects of automatic comet analysis using the Leitz MIAS image analysis system. The first step in validating the system was to prove its qualification for automatic comet analysis by establishing dose–response relationships using the standard mutagens ethylmethane sulfonate (EMS) and 2-aminoanthracene (2-AA) in V79 cells. In a second step, system reliability and usefulness for routine testing were evaluated. The aim was to establish a correlation between the analysis results of the Leitz MIAS system and the results of the Comet Assay II system (Perceptive Instruments), by using test compounds of distinct structural classes and various cell types.

Materials and methods

Comet assay

For the Comet assay we used the standard procedure originally described by Singh *et al.* (1988) with modifications (Klaude *et al.*, 1996; Hartmann *et al.*, 1998, Tebbs *et al.*, 1999). Regular slides were coated with 1.5% agarose and allowed to dry overnight. Cells were suspended 1:10 in 0.5% low melting point agarose (Sea Plaque GTG; FMC, Rockland, USA). Forty-five microliters of this suspension were spread on a precoated slide, covered with a 25×25 mm coverslip and placed at 4°C for 5 min. Two samples were placed on each slide as described by Tebbs *et al.* (1999). The coverslips were gently removed and the slide was submersed in lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100, pH 10) for at least 1.5 h. After lysis the slides were equilibrated for 20 min in alkaline buffer (300 mM NaOH, 1 mM EDTA, pH > 13, 4°C). Afterwards the slides were transferred to an electrophoresis unit with fresh alkaline buffer (4°C) and subjected to an electric field of 0.86 V/cm for 20 min. Following electrophoresis the microgels were neutralized in 0.4 M Tris, pH 7.5, rinsed with water, dehydrated in 100% ethanol for 2 min and allowed to dry at room temperature. The DNA was stained with 2.5 µg/ml propidium iodide. To prevent slides from fading and drying out, propidium iodide was first dissolved in distilled water and then

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further dissolved 1:5 in Vectashield (Vector Laboratories, Burlingame, USA). Interactive examination of coded slides was by image analysis (Comet Assay II; Perceptive Instruments, Haverhill, UK), using the tail moment as the measure of DNA damage.

Test compounds
For the first validation step with serial dilutions of standard mutagens in V79 cells the following compounds were used: EMS (Aldrich Chemical Co., USA) without metabolic activation; 2-AA with metabolic activation (10% S9 fraction).

To investigate the reliability of automatic comet analysis with the Leitz MIAS system a comparison with the Perceptive Instruments system was performed, using test compounds that were synthesized in-house as drug candidates. The chemicals were dissolved in either Eagles' minimal essential medium or dimethyl sulfoxide (DMSO). When DMSO was used, the final concentration in the culture medium was 1%. Dose selection was based on cell viability and/or cytotoxicity. In the absence of cytotoxicity all compounds were tested up to precipitating doses or 10 mM or 5 mg/ml, whichever was lower. Solvent control experiments were carried out both in the presence and absence of 10% S9 mix. The S9 mix was prepared using standard conditions (Maron and Ames, 1983). A liver homogenate was prepared from 7–9-week-old male Wistar rats [CRL:WI (GLX/BRL/HAN) IGS BR; Charles River, Sulzfeld, Germany]. Five days before being killed the animals were treated with a single i.p. injection of 500 mg/kg Aroclor 1254. The S9 fraction consisted of the supernatant obtained after centrifugation of the liver homogenate for 10 min at 9000 g and was stored at –70 to –85°C until needed. S9 mix, prepared with test tablets (Ames; Boehringer Mannheim, Germany), contained 10% S9 fraction, 4 mM NADP, 5 mM glucose 6-phosphate, 100 mM Na/K phosphate buffer, pH 7.4, 8 mM Mg aspartate and 33 mM KCl.

Cell types used
For our study V79 Chinese hamster and mouse lymphoma L5178Y cells were cultivated under standard conditions. For the tests with human leukocytes peripheral whole blood was collected and treated with the test compounds in RPMI medium.

Cytotoxicity assays
The test substance concentrations for the Comet assay were determined in a cytotoxicity test using a colorimetric method with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Sigma). The test was performed in 96-well cell culture plates. Cells were treated for 3 h, with or without S9 mix. Forty-eight hours after the start of treatment MTT solution (5 mg/ml in phosphate-buffered saline; Oxoid) was added to the wells. Two hours later the supernatant was removed and DMSO was added (100 µl) to extract the blue color. The plates were then evaluated spectrophotometrically at 570–690 nm. The test substance concentration leading to a reduction in staining intensity of ≥25% of the solvent control was used as the top concentration. For the Comet assay between four and six concentrations of a compound were used. For L5178Y cells Trypan blue dye exclusion was used as a measure of viability. Test compound concentrations resulting in ≤30% Trypan blue-positive cells were analyzed. Prior to analysis the slides were screened for cells with completely fragmented chromatin (hedgehogs or clouds) (Hartmann and Speit, 1997). Only samples which showed ≤15% hedgehogs were evaluated.

Comet analysis
For each concentration of a compound two independent cultures were used. The median tail moment of 50 cells/slide (1 slide/culture) was assessed and the average of the two cultures was calculated. For each compound at least three concentrations per experiment were analyzed. The results were confirmed in a repeat experiment. Generally, a compound was considered positive if the tail moment of at least one concentration with acceptable cytotoxicity was at least twice the corresponding tail moment value of the concurrent solvent control. No formal statistical analysis of the data was carried out. Instead, descriptive parameters, such as an obvious concentration-related increase in the tail moment, or the reproducibility of a specific effect were used for assessment of the data.

Automatic image analysis system
For automatic evaluation of the slides a Leitz MIAS image analyzer (Leitz Messtechnik, Wetzlar, Germany) together with a Leica DM RBE microscope (Leica Microsystems, Wetzlar, Germany) was used. The Leitz MIAS (Modular Image Analysis System) is a computer system using the UNIX-compatible operating system IDRIS with a special hardware architecture for real time image analysis. Morphological transformations on digital images can be efficiently performed by the pipeline architecture of the system, which uses additional video buses besides the standard VME bus. The 68030 CPU is supplemented by boards for image digitization, video display and image storage and dedicated boards for fast morphological gray and binary processing of images.

Image capture was done with a COHU 4912 B/W CCD camera mounted on the microscope equipped with motorized focus, an electronically driven eight slide stage and epifluorescence illumination for green excitation. For comet evaluation a Leica 40× PL APO objective was used.

The software for automatic comet analysis was exclusively developed in-house. It consists of three separate programs: a data input program for specification of the slides to be analyzed; an automatic measurement program; an optional relocation program which enables the operator to retrieve all measured comets for visual inspection.

Image analysis process
The evaluation task consisted of measuring 50 comets/sample. Damaged or otherwise odd-shaped nuclei, particles of cells with completely fragmented chromatin (due to apoptosis or cytotoxic effects) or nuclei that touched the image frame had to be excluded from scoring.

Finding valid nuclei for comet analysis
For image evaluation scanning of non-overlapping, adjacent image frames in a spiral scanning mode, starting at the center of a specimen, was performed. For each stage position a digital 512×512 image with 256 gray values was used. Autofocus was only performed if there were enough 'bright' signals, indicating one or more nuclei in the image. This was tested with a simple gray threshold and object analysis of the resulting binary image. If no sufficiently large bright object was present, the stage moved directly to the next image field. Otherwise, autofocus was performed. Due to the high quality of the optical equipment and the fluorescent staining in combination with antifade (Tebbs *et al.*, 1999), no specific 'fluorescence' autofocus had to be developed (Böcker *et al.*, 1997b). Instead, our standard autofocus algorithm, based on determination of the image with maximum contrast by moving the stage in the *z*-direction while capturing a series of images and analyzing them, was used, which has been proved to work efficiently for other automated applications (Frieauff *et al.*, 1998).

After autofocus and image capture, image analysis for a single image field consisted of the following processing steps. An initial thresholding of the original gray image with a fixed threshold which had been determined empirically to optimally segment the nucleus was performed first. After some morphological opening and closing for removal of small bright noise particles (including comet tail particles!), as well as closing small gaps of nucleus segmentation, an individual analysis of the segmented binary objects was carried out. For details on these morphological operations see Serra (1986). Considering metric parameters such as area and shape, as well as features like 'touching the image frame' or 'distance to other large, bright objects', each segmented object was classified either as 'valid nucleus for comet analysis' or as 'artifact to be rejected from further consideration'. Burst or overlapping nuclei as well as other debris were essentially eliminated at this stage. The coordinates of each accepted valid nucleus were stored for comet analysis at a later point in time. If no valid nucleus passed the metric analysis the current image field analysis was terminated and the next field was selected.

Comet analysis
After 50 valid nuclei had been detected the nucleus finding process was stopped for the current sample and the stage automatically returned to the first valid nucleus. For comet analysis each nucleus which had been previously accepted was positioned in the center of the image field. This guaranteed unbiased analysis of the comets without the influence of varying shading effects in different regions of the image field (due to uneven fluorescence illumination), as well as the complete comet within the current image.

An autofocus was again performed, but this time on a rectangular sub-range around the comet in order to maximize the contrast of the desired object alone. After image grabbing, threshold determination for comet segmentation was performed using a similar procedure to that described by Böcker *et al.* (1999). The optimal threshold was determined by successively decreasing the threshold from a starting value and counting the object number in the resulting binary image:

OBJ = Obj_Nr(Binary_Image(Current_Thresh)) – Obj_Nr(Binary_Image(Previous_Thresh))

where Binary_Image(Current_Thresh) denotes the binary image resulting from thresholding after having decreased Previous_Thresh, while Binary_Image(Previous_Thresh) was the binary image resulting from thresholding with the old threshold.

If OBJ exceeded a certain limit (200 particles in our case) we considered the computed threshold plus a constant, empirically determined offset to be the best threshold for comet segmentation.

The rationale of this approach was that through decreasing the threshold one approached the dark background, increasing noise particle segmentation, which led to a drastic increase in the number of objects in the image. So, before erroneously segmenting additional noise objects, the small comet tail particles could still be included. The gradient-like formula for OBJ yielded a

more robust and independent segmentation result of the actual number of comets in the image field as compared with a direct limit on the number of binary objects in the thresholded image, as described by Böcker *et al.* (1999).

After ensuring that the minimum distance of the centered nucleus to the next large, bright object (another nucleus or artifact) was exceeded, an analysis of the segmented objects characterizing the comet tail particles was performed. They should be located within a certain rectangular frame with the nucleus at one end and the distance between individual particles was not allowed to exceed a certain limit in order to discard bright particles not belonging to the comet.

The tail moment (TM) for the comet was computed by the following formula, a modification of the Olive tail moment (Olive *et al.*, 1990):

$$TM =$$

$$Relative\_Intensity \times (Nucleus\_Start - Nucleus\_Breadth - Tail\_Center\_Gravity),$$

with

$$Relative\_Intensity = Tail\_Intensity/Total\_Intensity$$

where Tail_Intensity denotes the integrated gray intensity values of the tail region, Total_Intensity denotes the integrated intensity of the whole comet, Nucleus_Start is the front end position of the nucleus (opposite of the tail), Nucleus_Breadth is the breadth of the nucleus (perpendicular to the direction of comet migration) and Tail_Center_Gravity denotes the average of the centers of gravity of the tail particles.

For retrieval and visual inspection of the measured comets by the relocation program their stage coordinates were stored together with the individual tail moments.

After all valid nuclei had been relocated and analyzed for comet tail moment the median over the resulting 50 individual tail moment values was computed to yield the overall descriptive parameter for the sample.

Experiments for the validation of automated analysis versus manual scoring

For validation of the reliability and usefulness of automatic image analysis for the Comet assay as a screening tool, automatic analysis was carried out in parallel with manual analysis (Comet Assay II; Perceptive Instruments). The first series of experiments consisted of treatment of V79 cells with various concentrations of standard mutagens. Furthermore, reproducibility of the test results was addressed by repeated analysis of slides with the automatic system. In a next step, in-house developed compounds with unknown genotoxic potential were used. These tests were conduced with V79 cells, L5178Y cells or human leukocytes. The aim was to clarify whether it was possible to clearly identify positive, weakly positive or negative effects with the MIAS system with the same sensitivity as the Perceptive Instruments system.

Results

Establishing dose–response curves with the different image analysis systems

For the first validation step V79 cells were treated with the direct acting mutagen EMS in the absence of S9 mix at 40, 100, 200, 330 or 400 µg/ml for 3 h. 2-AA was used with metabolic activation at concentrations of 17, 20, 21, 22 or 23 µg/ml. Fifty comets per sample were analyzed, using both the Leitz MIAS automatic system and the Perceptive Instruments Comet Assay II system.

The results for EMS treatment are shown in Figure 1A and B. For both systems a clear concentration-dependent increase in tail moment was found. The reproducibility of the results (repeated analysis of the same slides after 7 days) was very good. In the case of automatic Leitz MIAS only a slight decrease in tail moment was seen at high concentrations of EMS. This difference may be due to the prolonged storage, however, this does not change the overall picture. The results of two individuals scoring slides with the Perceptive Instruments system differ only marginally at higher concentrations of EMS and may reflect the influence of nucleus selection on the resulting tail moment values.

Figure 2 shows the results for the 2-AA-treated V79 cells. Similarly, a clear concentration-related increase in tail moment was found using both automatic Leitz MIAS and the Perceptive

A Leitz MIAS analysis

B Perceptive Inst. "Comet II"
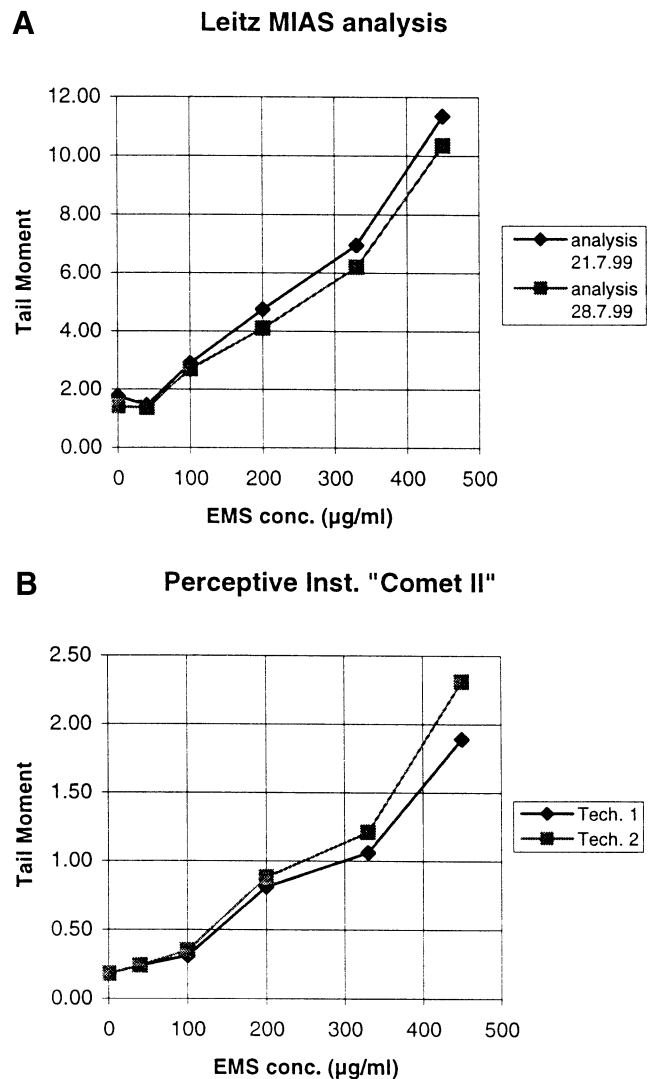
Fig. 1. (**A**) Comet assay analysis with the Leitz MIAS system: comparison of two different analyses at two distinct points in time for an EMS concentration series using V79 cells. (**B**) Comet assay analysis with the Perceptive Instruments system Comet II: comparison of two different analyses by two technicians for an EMS concentration series using V79 cells.

2-AA: MIAS vs Perceptive Inst.
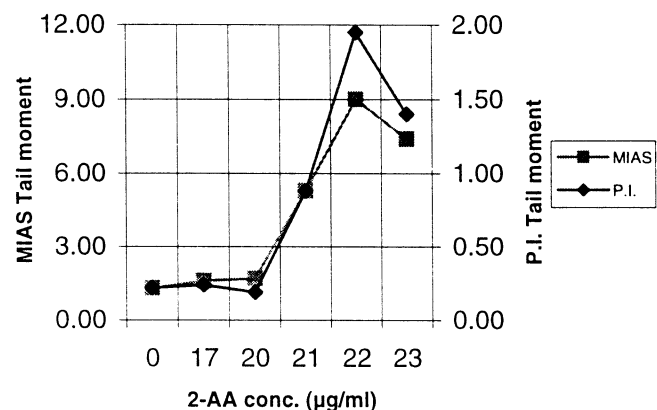
Fig. 2. Comparison of comet assay analysis using the Leitz MIAS and Perceptive Instruments systems: 2-AA concentration series using V79 cells.

Table I. Comparison of test results with the Perceptive Instruments and MIAS systems

Cell type	Compound	Treatment (µg/ml)	Tail moment MIAS	Tail moment Perceptive Instruments
V79	C1	0	2.15	0.40
		13.5	3.15	0.36
		18.9	2.35	0.45
		26.5	1.75	0.44
V79	C2	0	1.90	0.25
		83.3	2.05	0.20
		141.6	2.30	0.38
		240.8	1.30	0.21
		409.3	1.20	0.22
V79	C3	0	2.10	0.21
		105	1.75	0.32
		190	1.40	0.22
		342	1.45	0.29
		616	2.30	0.22
V79	C4	0	1.25	0.21
		6	1.30	0.22
		5	1.40	0.24
		4	**2.85**	**0.53**
V79	C5	0	0.95	0.16
		5	1.45	0.27
		4	**2.90**	**0.58**
		3	**2.95**	**0.51**
Leukocytes	L1	0	6.70	1.24
		120	10.80	1.92
		250	11.75	1.45
		310	11.20	2.21
		490	**16.10**	**2.67**
LY5178	EMS	0	5.25	0.77
		50	5.90	1.18
		100	**11.00**	**1.95**
		150	**11.50**	**2.18**
		200	**12.15**	**1.79**

All values are averages of two parallel cultures; 50 cells per culture were assessed. Positive effects are displayed in bold. The values for positive controls (performed in each test) are not shown.

Instruments system. Similar data were found using L5178Y cells treated with EMS (data not shown).

Automatic versus interactive comet analysis

Table I shows the results of the second validation step. V79 cells were treated with five Novartis screening compounds. The compounds showed different DNA damaging potential in the comet assay: three were clearly negative, one was positive only at the highest non-toxic dose and one was clearly positive. Additionally, one representative test with a compound tested in human leukocytes is shown. Both systems detected a positive effect only at the highest concentration. L5178Y cells were treated with EMS and positive effects were detected at the same concentration with both systems. Overall, in all cases concordant results with respect to positivity of the test compounds were achieved. These results confirm that automatic analysis is well-suited to identifying positive effects. In Figure 3 50 individual results of 10 tests are presented to demonstrate the high correlation of data gained with both systems using V79 cells, L5178Y cells and human leukocytes. Each data point in the graph represents the mean tail moment of a sample (consisting of two parallel cultures). The correlation is expressed by $y = 0.17x$, with a correlation coefficient of 0.9763.

The 6-fold increase in tail moment measured with MIAS as compared with the Perceptive Instruments values can be explained by the different frame grabbers of the two systems,

Comparison MIAS vs Perceptive Inst.
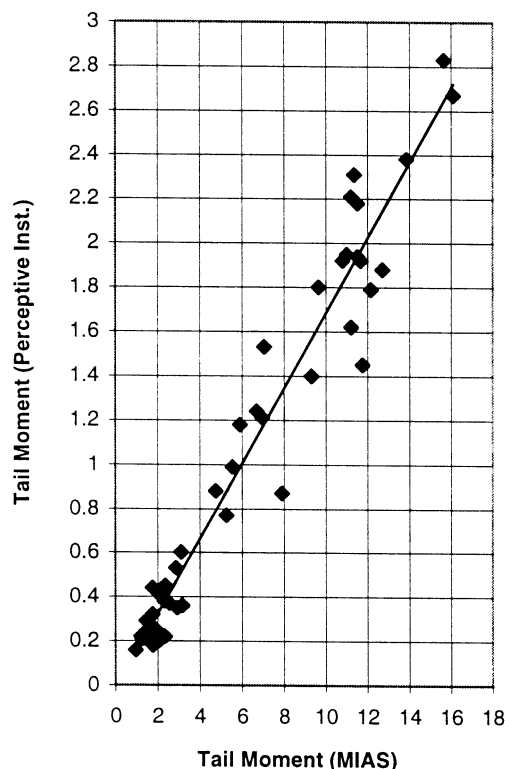
Fig. 3. Automatic versus interactive Comet assay analysis: tail moment values for 50 (pooled) compound concentrations.

microscopes with different optical components and, possibly, different implementations of tail moment computation.

Relocation of measured cells

Relocation of the analyzed comets showed that erroneous analysis of invalid comets (touching nuclei, clouds and otherwise misshapen objects) was no problem since computation of the median of tail moments meant 'ignoring' those few outliers which had no influence on the overall tail moment median of a sample.

Evaluation time

The interactive analysis of one sample (50 comets) with the Comet Assay II system (Perceptive Instruments) took ~10 min. As for automatic image analysis, the evaluation time depended upon the distribution density of nuclei on the slide.

The optimum comet density was one per image field on average. If there were more than four nuclei per field the minimum distance constraint for valid nuclei led to skipping of all comets and less than one comet per four image fields on average implied that a high number of 'empty' image fields had to be checked.

With the MIAS system the typical evaluation time for a standard sample with average cell density was 8 min for 50 comets (50–100 image fields to be analyzed). Sample specification and automatic analysis of 16 samples (i.e. eight slides) usually took <3 h, which made the time needed for automatic comet analysis comparable to interactive scoring.

Discussion and conclusion

The Comet assay is used in our laboratory as part of our screening test battery for the genotoxicity assessment of new

chemical entities. The aim of this study was to automate slide analysis for faster compound throughput, equivalent to screening more compounds in the same time.

We developed a computerized image analysis system for fully automated analysis of samples processed in the Comet assay. Our Leitz MIAS system can process up to 16 samples (eight slides, 2 samples/slide) without any user interaction. System performance proved to be independent of the cell type (V79 Chinese hamster cells, L5178Y mouse lymphoma cells and human leukocytes).

A series of tests for comparing interactive scoring with automatic image analysis showed that there was an excellent concordance between the results obtained with the two methods. Using the same criteria for a positive effect, the conclusions were the same in all experiments, irrespective of the scoring method and cell type used. Thus, automatic image analysis showed a high degree of reliability and well-known mutagens were easily detected. Weakly positive effects were detected with similar sensitivity by automatic image analysis as compared with manual comet scoring.

Since slide reading by image analysis is a fully automatic process, 50 samples/day can be easily analyzed. Little time is required for slide loading and sample specification. In summary, automatic image analysis using Leitz MIAS has proved to be an excellent system for fast, reliable and objective comet assay analysis for various cell types. The software package, consisting of programs for slide-specific information entry, automatic detection and optional relocation of the measured comets, has been shown to be reliable, efficient and user friendly. Since the Leitz MIAS system is no longer commercially available and due to faster PC image processing as well as more convenient PC software development tools, we recently decided to develop a robot-based, fully automated image analysis system based on the PC. This system, which is intended to be commercially available, will be the platform for a variety of automatic image analysis applications in toxicology.

Based on the results presented, we decided to replace interactive slide analysis by automatic slide reading in all our Comet assay experiments, conducted as screening tests in the genotoxicity assessment of potential new pharmaceuticals. This decision has led to a doubling in the throughput of compounds screened per technician, as compared with interactive scoring.

Acknowledgements

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