Development of a procedure for High Content and Higher Throughput Analysis of DNA-damage based on the COMET-Assay

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Situation

The COMET- or SCGE (single cell gel electrophoresis) Assay is a wide-spread method for determination of genotoxicity on the DNA level both *in vivo* and *in vitro*. The test is based on an electrophoresis of individual cells in a matrix of agarose after cell-lysis and unwinding of the DNA. In the electric field, the DNA migrates towards the anode leading to a higher migration rate for shorter fragments of DNA. After fluorescence staining, cells with DNA strand breaks appear in the shape of a "Comet", consisting of a "Comet head" which represents the undamaged DNA content of the nucleus and a "Comet tail", standing for the amount of DNA fragments.

The COMET-Assay is gaining more and more importance in genotoxicity testing. Since the first steps in development of this method by Rydberg and Johanson (1978) and Ostling and Johanson (1984), many improvements have been introduced with respect to (1) the specificity and sensitivity of the assay, (2) methodical variations to discriminate different types of DNA-damage such as single- and double strand breaks and modifications of DNA bases
(3) the knowledge about the importance of factors leading to false-positive results like cytotoxicity or apoptotic events (4) the use of the assay in combination with other endpoints related to genotoxicity such as micronuclei or chromosomal aberration and (5) efforts to introduce guidelines for the conduction of the assay in vivo and in vitro (Tice 2000, Hartmann 2003).

The practical procedure of the assay in its mostly used version is shown in figure 1 (left). Cells from various sources (*ex vivo*, suspension cells or adherent cells *in vitro*) are transferred to agarose coated standard slides, processed, including lysis, DNA-unwinding, electrophoresis and staining steps and delivered to microscopic examination. Mostly, the microscopic work is conducted in a semi-automatic way meaning that individual cells to be scored are manually selected by the user and analyzed by image analysis using appropriate PC-software.

With the increasing knowledge about the reliability and relevance of the COMET-Assay for early genotoxicity screening of drug candidates and for regulatory toxicology testing in vitro and in vivo, there is a need for more automated versions of this method leading to higher throughput systems.

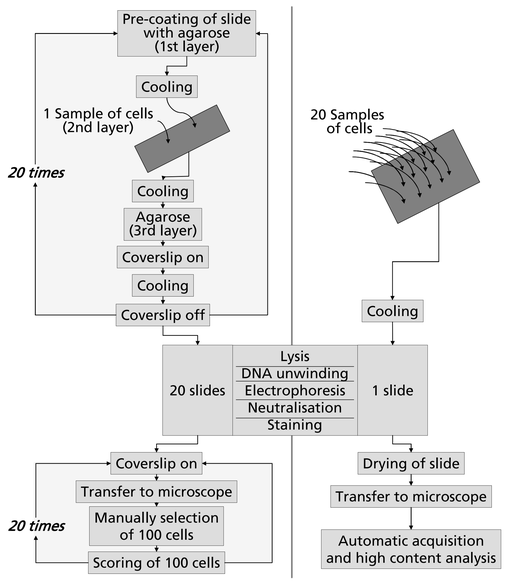

Figure 1 – Slide preparation, processing and analysis using the standard (left) and optimized (right) COMET procedure

Problem

Whereas the lysis, DNA-unwinding, electrophoresis and staining steps of the procedure can easily be transferred to automatic procedures, the concept of the use of single standard slides for individual COMET samples as well as the semi-automated procedure for COMET scoring are time consuming steps which prevent unattended conduction of the assay and higher throughput systems.

In the past, there have been several approaches to improve the sample situation (Kiskinis, 2002; Witte 2007) and to introduce methods for automatic scoring of comets (Böcker, 1999; Frieauff, 2001) using image analysis. However, there was no system available which enabled both the work with a more appropriate solution for the slide situation and at the same time an automatic scoring system.

Solution

An integrated procedure is being developed based on the following characteristics:

- Newly designed COMET-slides for the use of multiple individual spot gels
- Use of Olympus Scan^R High Content Screening Station
- Preservation of complete compatibility to the standard COMET method and flexibility to integrate any variations of the assay including discrimination of the neutral/alkaline version and work with repair enzymes such as FPG
- Extendable to higher throughput versions

Results

Preparation of slides

Glass slides were designed which enabled the work with 20 single spots on one slide and reduced the work for preparation of the COMET samples fundamentally. Figure 1 compares the standard and the optimized method of the assay. After addition of samples, the standard assay needs 5 steps to the transfer of the slides to the lysis buffer, the optimized version only needs one cooling step for hardening of the agarose. No coverslips are necessary.

Processing of slides

The processing of the slides remains unchanged compared to the standard assay. It can be performed under varying pH conditions to analyse for example single and double strand breaks as well as alkali labile sites (alkaline COMET version) or double strand breaks only (neutral COMET assay). The processing with repair enzymes such as FPG or hOGG can be easily included as well for analysis of specific damage of DNA bases.

After staining with standard DNA stains such as SYBR Green, the gel spots on the slide are dried and stable at least for 6 months (Woods, 1999). Hence, the following image acquisition does not have to be performed directly after slide preparation and COMET processing and storage enables the possibility of rescanning after time if needed.

Image acquisition

Slides are transferred to the microscope and subject to automatic image acquisition and analysis. The data acquisition procedure is performed as indicated in figure 2 (left) including a fast software auto-focus control and two channel image-acquisition to prevent image overexposure resp. saturation of the color channel. Hence, an acquisition of the full range of light intensities of the COMET fluorescence is guaranteed.

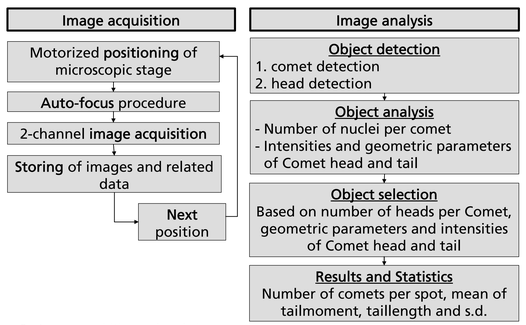

Figure 2 – Image acquisition and analysis process

Image analysis

Image analysis of the COMET samples can be done in real-time in parallel to the data acquisition on the same or a second personal computer. As well, the stored images can be analyzed later on.

The image acquisition procedure is indicated in figure 2 (right). It consists of 4 parts being processed in serial for each COMET gel spot on one slide.

During **object detection**, the image areas of single COMETs are defined and COMET heads are located by image analysis tools like definition of thresholds and location of relative increases of light densities. Figure 3 shows typical regions of interests (ROIs) from the object detection procedure where COMETs with lesser or more intensive effects were located.

Figure 3 – ROIs for whole COMETs and COMET Heads. Examples with increasing effect starting from control (left) to cells with considerably damaged nuclear DNA

The objects are analyzed with respect to geometric parameters such as length, width, perimeter, numbers of nuclei per comet, and light intensities (**object analysis**).
Based on these data, clear criteria are defined by using FACS-like scatter diagrams to select those objects which can be counted as COMETs and to reject objects like particles from staining, dust, cells near to the edges of the images or too close to other cells during **object selection**.
For the remaining objects, **results and statistics** can be assigned to treatment groups or to single gel spots on the slide. Standard results include the calculation of taillength and tailmoment as well as the number of counted COMETs per group or spot but can be easily extended to any other parameter which can be calculated from any geometric or intensity measurement of the COMET objects. Any individual object can be relocated on the spot, on the image and in the scatter diagrams. Treatment groups, single spots or manually selected groups of objects can be displayed in image galleries (figure 4).
By this way, a **clear and comprehensive control and documentation of all scored COMETs and any individual scored COMET is assured** all through the process of the COMET scoring, including image acquisition and –analysis.

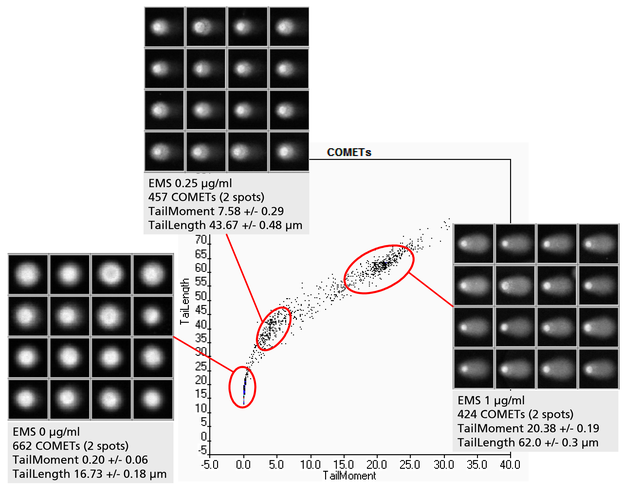

Figure 4 – Example of COMET results. A549 cells were exposed to Ethyl methanesulfonate (EMS) for 1 hour. Scatter diagram with taillength versus tailmoment for each individual cell from 3 concentrations (0, 0,25 and 1 µg/ml). 6 Spots on one slide were analyzed, 2 for each concentration. 16 COMETs from the gallery of all COMETs analyzed are shown as an example for each concentration.

Performance

In comparison to the standard COMET-Assay, less cells per sample are needed, more cells per gel are scored and times for the preparation of slides and scoring of the COMETs are significantly reduced (table 1). However, an even higher throughput can be realized using more spots per gel and roboter-handling throughout the procedure (see Future developments)

	Standard Assay	Optimized Assay	
No. of cells per sample	~ 100.000	~ 15.000	
Time for proc. of 20 samples	1 working day (8h)	preparation of slides:	5 min.
		scoring:	30 min.
Cells typically scored	50 or 100	at least 150 – 200	

Table 1 – Characteristics of the COMET assay versions

Future developments

Future developments of the method can be focused on the following topics

- **Implementation of roboter-handling procedures** to enable an unattended analysis of the COMET assay. The roboter handling would manage the slide preparation on the one and/or the transfer of slide to the microscope on the other hand.
- An **increased throughput** can be realized by minimizing the spot size and introduction of slides containing about 100 spots per slide. For the application of the samples on these slide a roboter-handling procedure is necessary.
- The contribution of cytotoxicity and apoptotic events to the effects quantified by the COMET assay is continuously being discussed. For control of such important aspects an additional step can be included in the method (figure 5) to **analyze cytotoxicity, apoptosis and the COMET assay on each individual cell** (Morley, 2006).
- For use in regulatory toxicology, a **processing in compliance to Good Laboratory Practice (GLP)** can be realized.

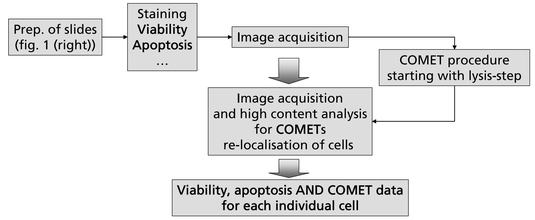

Figure 5 – Implementation of cytotoxicity and apoptosis measurements in the optimized COMET procedure

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