

Combining a Novel Comet Assay Procedure with Automated Imaging and Analysis to Enable High Throughput Genotoxicity Testing



Brad Larson¹, Clare Whittaker², Sandra Woodgate², and Peter Banks¹

¹BioTek Instruments, Inc. | Winooski, VT | USA • ²Trevigen, Inc. | Gaithersburg, MD | USA



Introduction

Human contact to genotoxins is inevitable, as agents detrimental to our DNA are present in our environment, and also a result of our own cellular metabolism where they can contribute to cell death, gene mutations, and even cancer, disease and aging^{1,2,3,4}. At the same time, therapeutic approaches such as chemotherapy and radiation treatment rely on initiation of tumor cell genotoxicity to produce positive therapeutic outcomes⁵. Efficiently and precisely repairing damaged DNA depends on the cellular DNA repair capacity. Therefore, quantifying DNA damage and accurately measuring the rate and extent of repair is critical in epidemiology, toxicology, drug development and other applications.

The comet assay is well established for quantifying DNA damage in mammalian cells and is compatible with detection of a wide array of DNA damaging agents. In fact, it can be used to monitor the cellular DNA repair capacity for several different DNA repair pathways. The assay's principle is migration of fragmented DNA in an electrophoresis gel, where intact DNA (comet head) moves at a slower rate than fragmented DNA (comet tail) as evaluated via fluorescence staining and microscopy⁶. The percentage of fragmented DNA in the comet tail is a direct measure of DNA damage.

Comet assays are typically performed and analyzed manually, so although the assay provides increased sensitivity, adoption as a reliable method has been slow. Here we demonstrate a combined process to generate automated, high-throughput comet-based genotoxicity data. High-density slides allow 96 individual samples to be compared simultaneously. Following staining, automated imaging and analysis of each well is performed using a novel cell imaging multi-mode reader with commonly accepted calculations; such as percent DNA in the tail. The combination of a novel single-cell microarray comet assay format^{7,8} and automated instrumentation and analysis, provides an accurate, robust method to assess DNA damage and DNA repair in mammalian cells.

BioTek Instrumentation

Cytation™ 5 Cell Imaging Multi-Mode Reader. Cytation 5 is a modular multi-mode microplate reader combined with automated digital microscopy. Filter- and monochromator-based microplate reading are available, and the microscopy module provides up to 60x magnification in fluorescence, brightfield, color brightfield and phase contrast. With special emphasis on live-cell assays, Cytation 5 features temperature control to 65 °C, CO₂/O₂ gas control and dual injectors for kinetic assays. Integrated Gen5™ Data Analysis Software controls Cytation 5. The instrument imaged stained DNA on the slides and CometChip using the GFP imaging channel. A slide adapter held the 2 well and 3 well CometSlides, and a two-part adapter was used when imaging the 96- well CometSlide and CometChip to ensure consistent positioning.

Materials and Methods

Assay and Experimental Components Alkaline CometAssay® Control Cells (Catalog No. 4256-010-CC), LMAgarose (Catalog No. 4250-050-02), 2-well CometSlides™ (Catalog No. 4250-004-03), 3-well FLARE™ Slides (Catalog No. 3950-075-02), 96-well CometSlides (Catalog No. 4253-096-03), Lysis Buffer (Catalog No. 4250-050-01) and CometAssay Electrophoresis System (Catalog No. 4250-050-ES) were donated by Trevigen, Inc. (Gaithersburg, MD). 96-Well CometChip® (Catalog No. 4260-096-01) and SYBR® Gold Nucleic Acid Gel Stain (Catalog No. S-11494, ThermoFisher, Carlsbad, CA), diluted to 1x, were also supplied by Trevigen, Inc.

96-Well CometChip System The 96-Well CometChip System (Catalog No. 4260-096-CS) from Trevigen simultaneously treats and measures DNA damage to one or more cell types on a single slide using the comet assay. It consists of a carrier base, macrowell former, lid and key and is suitable for use in tissue culture incubators. The CometChip is inserted into the carrier base, and when the macrowell former is magnetically snapped onto the base, it forms 96 sealed macrowells on top of the CometChip. Once treatment is complete, the standard alkaline comet assay is performed, resulting in a high density of non-overlapping cells for each macrowell.

Standard Alkaline Comet Assay Slides were prepared as follows or obtained directly from Trevigen. The standard alkaline comet assay was performed using Alkaline CometAssay Control Cells (CC), treated with increasing, proprietary concentrations of etoposide, embedded in low melting agarose and immobilized on 2-, 3- and 96-well specially treated slides to promote adherence. Slides were immersed in Lysis Buffer for 30-60 minutes at 4 °C, followed by a 30-minute room temperature equilibration in pH13 alkaline electrophoresis buffer. Alkaline electrophoresis was performed for 30-45 minutes at 4 °C and 1V/cm using the CometAssay Electrophoresis System. Slides were neutralized and agarose dried down before staining the DNA with SYBR Gold. Dry images were analyzed using the Loats Automated Comet Assay Scoring System (Loats Associates, Inc., Westminster, MD) at 10x magnification and compared to images obtained with Cytation 5 and integrated Gen5 Data Analysis Software.

Alkaline Comet Assay using CometChip Cultured lymphocyte cells in complete media were added to each CometChip well and captured into 30 µm micropores in agarose. Excess cells were aspirated, leaving a non-overlapping cell array. Multiple experimental conditions were performed in parallel by adding increasing etoposide concentrations in complete media and 30-minute treatments in the incubator. After media aspiration, CometChip was removed from the cassette, rinsed, overlaid with low melting agarose, and processed using standard alkaline comet conditions. CometChip was immersed in Lysis Buffer for 60 minutes at 4 °C, followed by a 40-minute, 4 °C equilibration in alkaline electrophoresis buffer. Electrophoresis was performed for 55 minutes at 4 °C and 1V/cm using the CometAssay Electrophoresis System. CometChip was neutralized before DNA staining with SYBR Gold. As the 96-well CometChip contains a wet agarose gel, the CometChip was oriented gel-side up when placed in the two-part adapter. These imaged comets appear in a mirrored configuration compared to those obtained using the CometSlide, however, this does not affect analyses. Wet images were obtained and analyzed with Gen5 Data Analysis Software and compared to data from 4x tiff wet well images analyzed with ImageJ open source software and the OpenComet plugin (www.opencomet.org).

2- and 3-Well CometSlide Imaging

Accurate automated comet imaging was validated using CometAssay Alkaline Control Cells containing known levels of DNA damage, processed using the standard alkaline comet assay on 2- and 3-well CometSlide slides. All were automatically imaged using previously determined offsets, eliminating manual determinations. The slides were placed in the slide adapter after staining with the stained wells facing down, in a typical configuration. Using a 4x3 image montage, a 8165 x 7956 µm area was captured from each well of the slides (Figure 1), yielding comet counts of 400-500 per well.

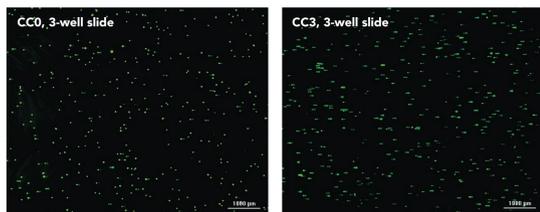


Figure 1. Well imaging. Stitched images of stained comets from single well using cryopreserved Alkaline CometAssay Control Cell population CC0 (healthy control population) and CC3 (healthy control population treated with high etoposide concentration).

2- and 3-Well CometSlide Imaging (cont.)

Exposure parameters were set such that fluorescence values per pixel were within the CCD camera quantifiable range. Auto-focus on all individual images in the montage was also performed (Figure 2).

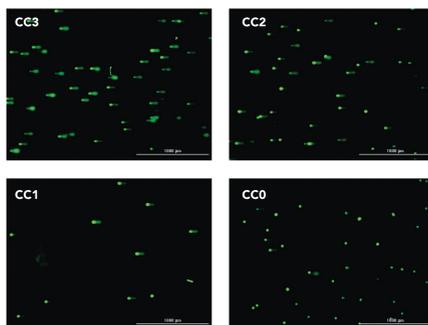


Figure 2. 3 well CometSlide 2.5x images. Images captured from individual wells of Alkaline CometAssay Control Cell populations, revealing high (CC3), medium (CC2), low (CC1) or no appreciable DNA damage above normal levels (CC0).

The signal was accurately captured from comets containing no DNA damage, with fluorescence emanating solely from the comet head, as especially demonstrated in Figure 2 CC0; and also from comets containing high DNA damage, where fluorescence was distributed between the comet head and tail, as especially demonstrated in Figure 2 CC3.

Percent DNA in Tail Calculation

Two separate, automated cellular analyses determined “Percent DNA in the Tail”, taking into account the change in comet circularity when DNA was damaged by a test agent. Undamaged comet DNA remained a consistent length, and during electrophoresis, little to no migration was seen; the comet appeared as a highly circular green dot (Figure 2 CC0). In comparison, highly damaged DNA consisted of fragments, in addition to that remaining undamaged. Following electrophoresis, smaller fragments migrated farther than larger ones, creating a comet shape, with the comet head containing undamaged DNA and the comet tail containing smaller fragments created by the damaging test agent (Figure 2 CC3). The circularity of these two portions, when analyzed as one object, decreased as a function of the extent of the comet tail; and by proxy, the genotoxicity level. The first cellular analysis step determined total comet circularity. Primary and advanced criteria (Table 1) were set to automatically place object masks around each entire comet, despite the genotoxicity level (Figure 3).

Total Comet Primary Cellular Analysis Parameters		Total Comet Head Primary Cellular Analysis Parameters	
Threshold	4,000 RFU	Threshold	35,000 RFU
Min. Object Size	25 µm	Min. Object Size	2 µm
Max. Object Size	200 µm	Max. Object Size	75 µm
Bright objects on a dark background	Checked	Bright objects on a dark background	Checked
Split touching objects	Unchecked	Split touching objects	Unchecked
Advanced Options		Advanced Options	
Evaluate Background On	5% of Lowest Pixels	Evaluate Background On	5% of Lowest Pixels
Image Smoothing Strength	0	Image Smoothing Strength	1
Background Flattening Size	50 µm	Background Flattening Size	40 µm
Total Comet Sub-population Parameters		Total Comet Sub-population Parameters	
Standard Deviation	< 25,000 RFU	Circularity	< 0.05 RFU
Standard Deviation	> 2000 RFU	Size	≤ 5 µm
Integral	< 6.0x10 ⁷ RFU		
Size	≤ 120 µm		

Table 2. Gen5 comet head cellular analysis parameters.

Table 1. Gen5 total comet cellular analysis parameters.

Additional sub-population criteria was applied (Table 1) to ensure that only true single comets were used in the final calculation. During the standard alkaline comet assay, nuclei may be in close proximity to each other, or overlap, causing multiple comets to be identified as a single object (Figure 3). These objects demonstrate criteria different than normal comets, so by setting standard deviation, integral, and size subpopulation parameters, multiple comets were eliminated from final analysis. Object masks also excluded cell fragments, lint, and other anomalies exhibiting a consistently high or low signal with little variation. By adding a final subpopulation metric to ensure that objects contain a certain signal variation, false objects were discarded. The final objects yielded results with a high degree of accuracy and consistency. The second cellular analysis determined comet head circularity. Primary and advanced criteria (Table 2) were modified such that object masks were placed around the comet head only. Sub-population metrics eliminated non-comet objects.

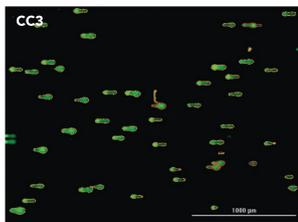


Figure 3. Total comet primary cellular analysis object masks. Object masks automatically placed around comets from CC3 Alkaline CometAssay Control Cell populations on 3 well CometSlides, using Gen5 primary cellular analysis parameters. Red highlighted objects eliminated from, and gold highlighted objects included in, additional calculations.

Using the final circularity data generated from the total comet and comet head analyses, percent DNA in the tail was determined. Gen5 used the following formula to automatically calculate the final value:

$$(1 - (\text{Total Comet Circularity} / \text{Comet Head Circularity})) * 100$$

The fraction represented the change in circularity from the total comet to the comet head, or the fractional DNA in the comet head. Percent DNA in the tail was calculated for duplicate 2 and 3 well slides, using each of the four Alkaline CometAssay Control Cell populations (CC0-CC3).

2 Well CometSlide % DNA in Tail Analysis				
Etoposide Treatment	Gen5 Analysis (n=4 Wells)		Loats Analysis (n=4 Wells)	
	Total Comet Count	% DNA in Tail	Total Comet Count	% DNA in Tail
CC0	367	0.24±1.40%	173	4.11±1.16%
CC1	328	18.65±2.89%	253	20.55±1.31%
CC2	297	46.64±3.20%	171	41.51±3.17%
CC3	407	55.08±2.45%	204	57.61±4.26%
3 Well FLARE Slide % DNA in Tail Analysis				
Etoposide Treatment	Gen5 Analysis (n=6 Wells)		Loats Analysis (n=4 Wells)	
	Total Comet Count	% DNA in Tail	Total Comet Count	% DNA in Tail
CC0	372	3.53±1.85%	177	3.52±1.72%
CC1	139	29.903.04%	79	23.39±3.16%
CC2	372	39.56±3.18%	178	38.35±2.87%
CC3	483	56.73±1.59%	199	60.18±2.91%

Table 3. Percent DNA in tail analysis for 2 well and 3 well CometSlides using CometAssay Control Cells in the standard alkaline comet assay.

As in Table 3, percent DNA in tail values for 2 well and 3 well CometSlides increased appropriately with increasing etoposide treatment (CC1-CC3) of the healthy population (CC0). Values were compared to those generated previously from the same slides using common comet assay methods, confirming that the automated imaging and analysis methods used by Cytation 5 delivered accurate results.

Comet Tail Moment Calculation

In addition to percent DNA in tail calculations, one of the most accepted comet evaluation methods is the comet tail moment, or tail moment. The method⁹ combined measurements of the comet head, tail (Figure 4), and the percent DNA in tail value.

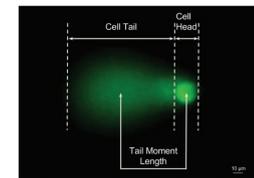


Figure 4. Comet areas included in comet tail moment calculation.

2 Well CometSlide Tail Moment				
Etoposide Treatment	Gen5 Analysis (n=4 Wells)		Loats Analysis (n=4 Wells)	
	Total Comet Count	Tail Moment	Total Comet Count	Tail Moment
CC0	367	0.02±1.17	173	0.41±0.17
CC1	328	5.53±0.96	253	4.34±0.33
CC2	297	10.71±1.04	171	10.66±1.01
CC3	407	19.54±1.66	204	16.88±2.19
3 Well FLARE Slide Tail Moment				
Etoposide Treatment	Gen5 Analysis (n=6 Wells)		Loats Analysis (n=4 Wells)	
	Total Comet Count	Tail Moment	Total Comet Count	Tail Moment
CC0	372	0.91±0.49	177	0.42±0.36
CC1	139	5.66±0.61	79	5.19±1.20
CC2	372	11.04±1.33	178	9.35±1.04
CC3	483	19.52±0.86	199	17.22±1.78

Table 4. Comet tail moment calculations for 2 and 3 well CometSlides.

During total comet and comet head analyses, object size calculations were performed in addition to object circularity, and used in the following formula to calculate comet tail moment:

$$(DS3 * ((DS1 - DS2) / 2) + (DS2 / 2)) / 100$$

Where DS1 was the total comet length; DS2 was the comet head length; and DS3 was the percent DNA in tail value. Comets exhibiting little to no DNA damage had a tail moment value approaching zero. As DNA damage increased, tail moment increased appropriately. Comparisons were made for comet tail moment values calculated using Gen5 and Loats Analysis software previously used for percent DNA in tail determinations (Table 4). Similarity in the values confirmed that Gen5 can also be used to calculate the commonly used comet tail moment value, in addition to percent DNA in tail.

96-Well CometSlide Imaging and Analysis

The 96-well CometSlide analyzes samples in a high-throughput format. Automated imaging was carried out using a single imaging read step. Auto-focus was incorporated, and exposure settings were as previously described. Imaging and analysis validation was carried out using a 96-well CometSlide and the Alkaline CometAssay Control Cells with twelve replicate wells for each control cell population. An average of 30-40 comets were captured in each 2.5x Cytation 5 image, and approximately 40 comets with Loats Analysis software at 10x magnification (data not shown). Analyses were performed (data not shown) using Gen5 and the aforementioned parameters.

Results were compared to previously generated data using the same slide and Loats Analysis software. Figure 5 illustrated how similar percent DNA in tail and comet tail moment values can be compared to other analysis packages and across replicates generated using a combined process that saves time compared to manual imaging and evaluation.

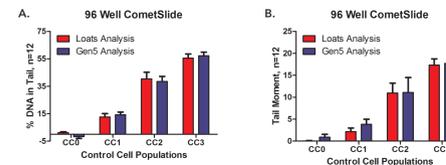


Figure 5. (A) Percent DNA in tail calculations and (B) tail moment for 96-well CometSlide using Alkaline CometAssay Control Cells in the standard comet assay.

96-Well CometChip Imaging and Analysis

The 96-Well CometChip System increases throughput as it allows simultaneously treatment and measurement of DNA damage induced by varying treatments, or among different cell types on a single slide. Agarose patterning creates even comet distribution throughout the well, increasing simplicity and accuracy. Automated imaging using the 96-well adapter was performed similarly to that using the 96-well CometSlide. Healthy lymphocyte cells (T0) were captured into micropores and twenty-four replicate wells were treated with increasing etoposide concentrations (T1-T3). After processing the CometChip in a similar manner to the 96-well CometSlide procedure, it was used to validate imaging and analysis. 100-120 comets were captured in each 2.5x image, where the micropores eliminated randomness of comet placement for simplified analysis and a reduced number of objects requiring removal prior to final calculations (Figure 6).

Final percent DNA in tail values, when compared to previously generated data from the same slide, demonstrate that Cytation 5 delivered reliable, accurate results with the 96-Well CometChip System.

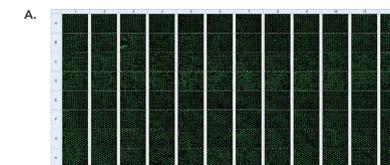
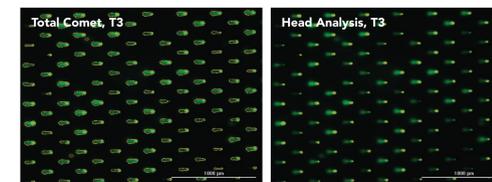


Figure 6. Automated 96-well CometChip imaging and analysis. (A) Image thumbnails from automated imaging of 96-well CometChip. Object masks automatically drawn around total comet or comet heads using Table 1 and 2 analysis parameters. Red highlighted objects eliminated from, and gold highlighted objects included in, final calculation.



96 Well CometChip % DNA in Tail Analysis				
Etoposide Treatment	Gen5 Analysis (n=24 Wells)		OpenComet Analysis (n=24 Wells)	
	Average Comet Count	% of DNA in Tail	Average Comet Count	% of DNA in Tail
T0	115	0.99±2.13%	131	6.38±2.04%
T1	114	33.33±4.88%	155	30.12±4.13%
T2	114	56.02±5.32%	160	52.34±7.81%
T3	114	67.81±6.04%	159	69.71±8.98%

Table 5. Percent DNA in tail calculations for 96-well CometChip.

Conclusions

- The alkaline comet assay is a robust method for genotoxicity measurements, and using the 96-well CometSlide and CometChip, analysis is efficiently performed in a high-throughput format.
- Automated comet imaging, using Cytation 5, is performed rapidly and with a high degree of clarity, while single or image montage capture is automated using all slide and CometChip configurations.
- Comet analysis using Gen5 Data Analysis Software allows essential calculations to be immediately and automatically determined, removing manual subjectivity and variability from final reported data.
- The combination of assays system, automated imaging and analysis method creates an easy to use, robust method to perform genotoxicity measurements on important test molecules.