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Short communication

Critical points in Comet assay silver staining procedure

Misko Milev, Viktorija Maksimova, Milkica Janeva, Tatjana Ruskovska*

Faculty of Medical Sciences, Krste Misirkov 10-A, 2000 Stip, North Macedonia

Introduction

Comet assay is one of the most common methods for measuring DNA damage in eukaryotic cells. In 1984, Ostling and Johanson demonstrated movement of DNA strands when nuclei were exposed to an electric field. Later, Singh et al. (1998) modified and optimized the method using alkaline conditions during electrophoresis, thus increasing specificity and reproducibility of the method. In the recent years, this assay has gained in popularity because of its simple, economical, versatile and sensitive procedure. This assay requires only a small number of cells per sample and provides collection of data at individual cell level, allowing robust statistical analysis. According to Azqueta and Collins (2013), there is a number of comet assay variations, applicable to different types of samples, such as: peripheral blood cells, cell lines, buccal mucosa, yeasts, cancer cells and plant cells.

This method can measure single-strand or double-strand DNA breaks, DNA cross-links, alkali labile sites, base/base-pair damages and apoptotic nuclei (Collins, 2004). It has widespread applications in the area of testing novel pharmaceuticals for genotoxicity, monitoring environmental contamination with genotoxins, human biomonitoring and molecular epidemiology, diagnosis of genetic disorders and fundamental research in DNA damage and repair. During electrophoresis the damaged cells acquire the shape of a comet, hence the cells are called comets and the procedure comet assay. These comets can be visualized using fluorescent staining method or silver staining method.

In this communication, we have considered silver stained comet assay images because they are preferred in clinical applications (Jackson and Bartek, 2009). This is mainly because fluorescent staining requires high quality fluorescence microscope. Further, with fluorescent staining the slides cannot be stored for a long period of time and they should be photographed and analyzed immediately. The advantages of silver staining method are that it is inexpensive, slides can be preserved for a long time, and the analysis can be carried out using a simple light microscope. The main disadvantage of silver staining compared to fluorescent images is that silver stained images have a high level of background noise, which should be diminished as much as possible. Therefore, we have identified the critical points of comet assay silver staining procedure, as established and optimized in our laboratory.

Materials and methods

Peripheral blood mononuclear cells were embedded in 0.7% LMA (low melting agarose) on a microscope slide (around 10^6 cells per slide) precoated with 1% NMA (normal melting agarose). Then cells were lysed at 4 °C for minimum of 1 hour. Lysis solution was prepared with NaCl (2.5 M), EDTA (100 mM) and Tris base (10 mM), pH=10. Before use, cold 1% Triton X was added. This procedure disrupts the membranes and removes cytoplasm and histones.

* tatjana.ruskovska@ugd.edu.mk

The remained coiled DNA, so called nucleoid, was incubated at 4 °C for 40 min at pH > 13 in the electrophoretic buffer (300 mM NaOH, 1 mM EDTA), thus allowing for DNA to unwind. A 300 mA current with voltage of 0.8 V/cm across the field was applied for 30 minutes, which allowed the damaged DNA parts to travel toward the anode.

After electrophoresis, slides were flooded 3 times for 5 minutes with neutralizing buffer (pH=7.4) and then with deionized water, after which the sides were allowed to dry for 1 hour at room temperature.

Then the slides were fixed for 10 minutes in fixative solution (1.5% *w/v* trichloroacetic acid, 5% *w/v* zinc sulfate and 5% glycerol). After fixation the slides were washed 3 times with deionized water and left overnight to dry at room temperature. Before silver staining slides were re-hydrated for 5 minutes in deionized water.

The silver staining solution was prepared fresh in following sequence: 34 mL of solution B (5% *w/v* sodium carbonate, 0.2% *w/v* ammonium nitrate, 0.2% *w/v* silver nitrate, 0.5% *w/v* tungstosilicic acid, 0.15% *v/v* formaldehyde) to 66 mL of Solution A (5% *w/v* sodium carbonate). After staining of the slides, they were washed 3 times with deionized water. The staining was stopped with a treatment of slides for 5 minutes at 1% acetic acid solution.

Results and discussion

As an alternative method for dyeing, the silver staining is more economical but requires much more time and experience. The optimization of silver staining method in single cell electrophoresis has shown many critical issues which should be strictly considered when applying this type of staining to the examined DNA:

1. High cleanliness of laboratory glass – all the glass material used, should be pretreated with 50% of nitric acid solution, then washed with detergents and deionized water.

2. Microscopic slides should be dried overnight at room temperature after coating them with NMA.

3. Time of silver staining – three series of 10 minutes with fresh solution should be performed on low intensity shaker until light gray color is formed. Slide orientation and position should be modified because waves in the staining solution can cause aggregations of silver particles.

4. Staining tray should be covered with aluminum foil to provide dark conditions.

5. Freshness of staining solution is another sensitive variable. The staining solutions should always be freshly prepared with intense mixing and with minimum exposure to intense light.

Other parameters, not specific to silver staining, should be also taken in consideration. Concentration of LMA, cell density, time of unwinding of DNA in high pH solution, voltage, time and temperature during electrophoresis can significantly influence the outcome of the assay, so they should remain constant.

Conclusion

Taking into account all these critical points, silver staining can produce excellent quantification of DNA damage with comet assay using only standard light microscopy.

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