IMPROVEMENT of *IN SITU* SLIDE PREPARATION PROCEDURE for SEMI-AUTOMATED MICRONUCLEUS COUNTING SYSTEM

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【Abstract】

The *in situ* micronucleus (MN) test is easier to use than traditional methods such as the dish approach. Nevertheless, the necessary resources for manual counting and advance preparation for reaching consensus of counting criteria between counters are the same for both procedures. Automated MN counting systems are effective tools to address many difficulties associated with manually counted MN tests. It is expected that the combination of *in situ* methods and automated counting systems would facilitate increased throughput.

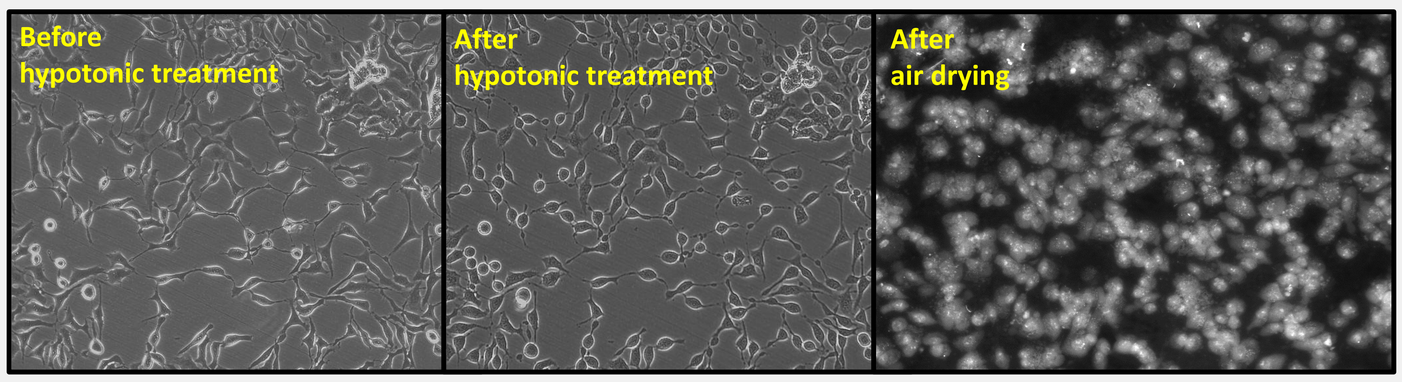
Algorithms of automated systems for image analysis have two major functions: cell outline recognition and **MN findings**. Adherent cells that are usually used for the *in situ* methods have infinite shapes, which complicates development of cell recognition algorithms. In addition, changing cell morphology caused by chemical effects amplifies these difficulties. This means that stabilizing cell morphology is key for success in developing such algorithms.

In this study, we conducted **several experiments to stabilize cell shapes.**

Hypotonic treatment is one procedure for stabilizing cell morphology. Although it is well known that hypotonic solutions change cells to a spherical shape in aqueous media regardless of culture conditions, **there are no reported methods for keeping cells in a round shape on dried slides** until the MN scoring step.

【Past Procedures for MN Counting】

Seeding	2 x 10^4 cells/well (Lab-Tek II CC2, 2 well), 24h	
TPM treatment	*XX* ug TPM/mL, 2% DMSO in medium, 3h (Short term)	
Medium Change	*Saline* wash x 1, 10% BS-MEM, 21h (Short term)	
Slide Preparation		
Hypotonic shock	0.075M KCl, 5 min.	
Pre-Fixation	Methanol:Acetic adid= 3:1, Add 10% v/v in medium, 5 min	
Fixation 1	Methanol:Acetic adid= 3:1, Complete replacement, 30 min	
Fixation 2	Methanol:Acetic adid= 99:1, Complete replacement, 5 min	
Air Dry		
Fluor-Stain	Acridine Orange	Nucleus: DAPI, Cytoplasm: Cell Mask Orange
MN Scoring	Manual Scoring	Automated



Dried cells on our slides did not have clear cell outlines and these cells were undetectable by the automated counting system (cell detection rate: 0.5–2.0% in the field of view, not shown).

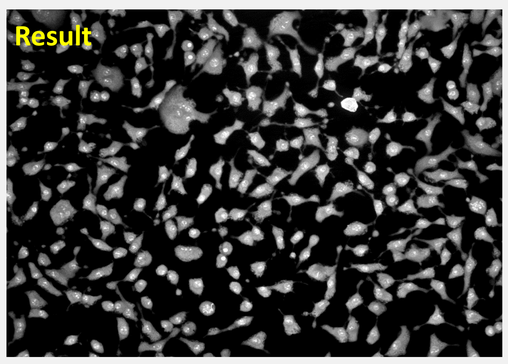
Note:

Although the protocol we used was based on the method of the chromosome aberration test, it was unsuitable for automated MN scoring, especially cell outline recognition, because this protocol was designed to disrupt the cell membrane for dispersing chromosomes on glass slides in the drying stage.

【Modification 1 : Deacidification】

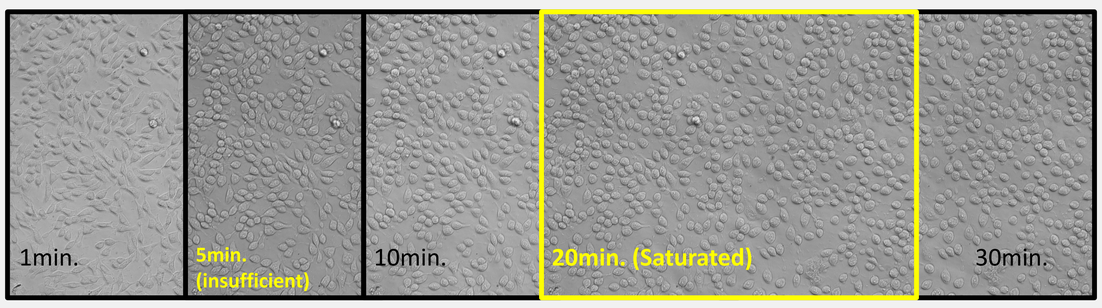
Acetic acid is thought to be a key constituent for lysing cells. If cells are fixed by only methanol after hypotonic treatment, the swelled cells are rapidly deflated (not shown); therefore, acetic acid was thought to be necessary at least during fixation.

Slide Preparation	
Hypotonic Shock	Same as above
Pre-Fixation	same as above
Fixation 1	same as above
Fixation 2	same as above
Deacidification	70% ethanol, Complete replacement, twice
Air Dry	



- **Almost no cells are lysed after air drying**
- **Cells have adequate space for expansion**

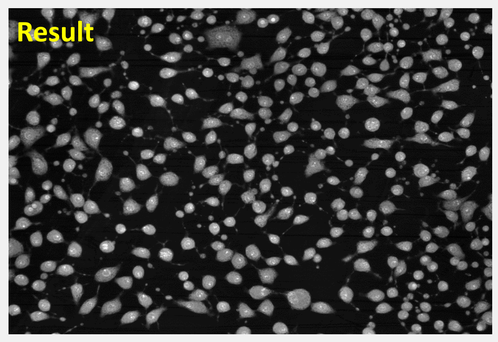
【Confirmation of Hypotonic Tolerance (Time Lapse) 】



Cells continued swelling in the hypotonic solution for up to 20 min.

【Modification 2: Extending Hypotonic Treatment】

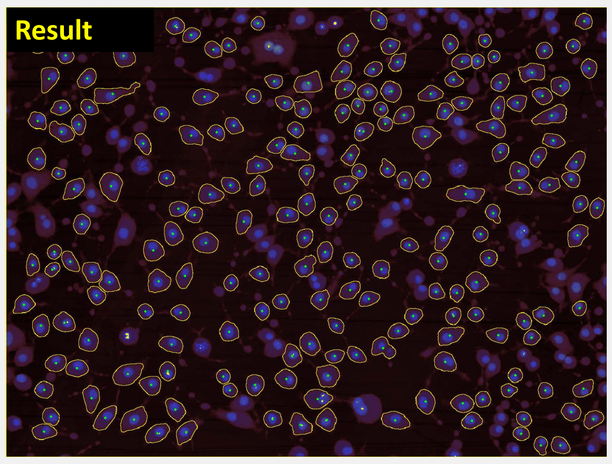
Slide Preparation	
Hypotonic Shock	0.075M KCl, 20 min.
Pre-Fixation	Methanol:Acetic adid= 3:1, 10%v/v in medium, instantly
Fixation 1	Methanol:Acetic adid= 3:1, Complete replacement, instantly
Fixation 2	Methanol:Acetic adid= 99:1, Complete replacement, instantly
Deacidification	70% ethanol, Complete replacement, twice
Air Dry	



Combination of long hypotonic treatment and deacidification was exceptionally effective for maintaining round cell edges.

【Verifications for Semi-Automated Counting System】

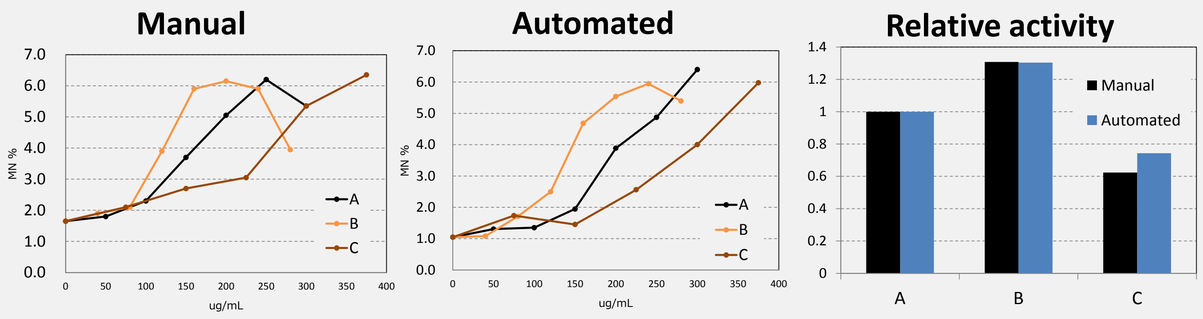
Cell Outline Recognition



Cell detection rates reached 60%–80% in all samples.

Note:
False negative rates of MN detection were <10% of total MNs in all samples. Although many false positive MNs were detected, these were eliminated by analysis with the Pathfinder System (IMSTAR S.A.).

Comparison Between MN Counting Methods



There were no differences between manual counting and automated scoring in evaluation of MN activity.

【Acknowledgment】 Papine A., Homsy C., Soussaline M., Soussaline F. (IMSTAR S.A., France)