Standard Operating Procedure @ Sciensano

PROTOCOL	***In vitro* micronucleus test in human keratinocyte HaCaT cells and 5G RF-EMF exposure**
DATE	08/04/2024
AUTHOR(S)	Seppe Segers, Roel Anthonissen
REVISED BY	Birgit Mertens
APPROVED BY	Birgit Mertens

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1 Objective and purpose

This procedure describes the method for the detection and automated scoring of micronuclei in HaCaT cells. Other cell types may be used, if justified. Depending on the characteristics of the cell type, modifications to the procedure may be required.

2 Definitions et abbreviations

CytoB	Cytochalasine B
DAPI	4', 6'-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
FBS	Foetal Bovine Serum
KCl	Potassium Chloride
MMS	Methyl Methanesulfonate
PBS	Phosphate Buffered Saline
RO	reverse osmosis

3 Procedure

3.1. Security

- Protective Clothing: Gloves, safety glasses and laboratory coat.
- All manipulations before the fixation of the cells (0) should be performed in a biosafety cabinet. Later manipulations involving chemicals should be performed under a chemical hood.
- All safety measures related to working with potentially carcinogenic chemicals should be respected. Depending on the test products, additional safety measures may be required (see Security sheet).

3.2. Controls

Positive control substances:

- Positive control substances active **without metabolic activation**:
  Methyl Methanesulfonate (MMS) 150 MMS

Alternative positive control substances can be used, if justified.

Negative control substance:

- Treatment medium

3.3. HaCaT Cell Line

HaCaT is a spontaneously immortalized keratinocyte cell line from the adult human skin of a 62-year-old male. Due to their high capacity to differentiate and proliferate in vitro, these cells are extensively utilized in skin biology and differentiation research.

3.4. Chemicals

Acetic Acid (VWR, Heverlee (Leuven), Belgium or equivalent)
Amphotericin B (Thermo Fisher Scientific)
Cytochalasin B (Sigma-Aldrich)
DMEM (Dulbecco's Modified Eagle Medium) (Thermo Fisher Scientific)
DMSO (Dimethylsulfoxide) (Sigma-Aldrich)
Ethanol (denatured) (VWR, Heverlee (Leuven), Belgium or equivalent)
Paraformaldehyde
FBS (Foetal Bovine Serum) (Thermo Fisher Scientific)
Gentamycin (Thermo Fisher Scientific)
Glutamax (Thermo Fisher Scientific)
Methanol (VWR)
MMS (Sigma-Aldrich)
KCl (VWR)
Non Essential Amino Acids (Thermo Fisher Scientific)

PBS pH 7.2 (Thermo Fisher Scientific)
Sodium Pyruvate (Thermo Fisher Scientific)
TrypLETM (Thermo Fisher Scientific)
Vectashield mounting medium containing DAPI (Labconsult, Brussels, Belgium or equivalent)

4 Solutions

4.1. Medium HaCaT cells

Complete medium: Add to a 500 ml DMEM bottle:

- 6 ml Gentamycin, Glutamax, Sodium Pyruvate & Non Essential Amino Acids
- 60 ml FBS (2 aliquots)
- 600 µl Amphoterecin B (1 aliquot)
- Label & store at 4°C for maximum 1 month.

4.2. Cytochalasine B (cytoB)

Stock solution (3.33 mg/ml):

- Dissolve 5 mg cytoB in 1.5 ml DMSO. Make aliquots of 25 µl and store them at -20°C protected from light for maximum 3 years.

Working solution (3 µg/ml):

- For 12 samples (25 ml):

$$C1\ x\ V1 = C2\ x\ V2$$

$$\mathrm{V1} = \frac{\mathrm{C2\ x\ V2}}{\mathrm{C1}}$$

$$\mathrm{v1} = \frac{3\,\frac{\mu g}{ml}\,\mathrm{x}\ 25\ \mathrm{ml}}{3333\ \mu\frac{g}{ml}} = 22.5\ \mu\mathrm{l\ stock\ solution\ in\ 25\ ml\ medium + FBS}$$

The working solution should be prepared freshly!

4.3. KCl solution (0.075 M)

- Dissolve 559 mg KCl in 100 ml RO water. Keep in a water bath at 35°C
- *The KCl solution should be prepared freshly!*

4.4. Fixator

4.4.1. Fixator 1

- Fixator 1 (MeOH:CH_3COOH in a ratio 3:1) is prepared just before use and should be kept ice-cold (4°C) throughout the whole fixation
- For 12 samples:
  40 ml CH_3COOH + 120 ml MeOH

  Keep at 4 °C

4.4.2. Fixator 2

- Fixator 2 (MeOH:CH_3COOH in a ratio 9:1) is prepared just before use and should be kept ice-cold (4°C) throughout the whole fixation
- For 12 samples:
  10 ml CH_3COOH + 90 ml MeOH

  Keep at 4 °C

4.5. Propidium Iodide

Stock solution (1 mg/ml)

- Prepare a 1 mg/ml Propidium Iodide solution in RO water and store at 4°C protected from light for maximum 1 year

Working solution (1.5 µg/ml):.

- Dilute the Propidium Iodide stock solution to 1.5 µg/ml with PBS
- The working solution should be prepared freshly
  For example (10 samples): 10 µl stock solution + 990 µl PBS = 10 µg/ml
  100 10 µg/ml + 900 µl PBS = 1.5 µg/ml

4.6. Positive Controls

4.6.1. MMS (200 µM)

Stock solution (184,6 mM):

- The solution should be prepared freshly
- Add 15.4 µl MMS to 984.6 µl of culture medium.

Intermediate solution (10 mM):

- Add 54,17 µl MMS stock solution to 945,83 µl of culture medium.

Working solution (422 µM):

- Dilute 372 µl MMS intermediate solution to 8800 µl with culture medium.
- When 1,1 mL working solution is added to a dish with 2 mL medium, the end concentration will be 150 µM.

5. Experimental procedure

Experiments are performed both in presence and absence of an exogenous metabolising system (i.e. S9 metabolic fraction) in order to detect also compounds requiring metabolic activation.

5.1. Slide cleaning (optional)

- Put slides overnight (or longer) in an acetic acid solution (10%).
- Rinse them in RO water.
- Move them to another jar containing denaturated alcohol for at least one night.
- Dry the slides by polishing them with a medical towel. They are now ready for use.

5.2. Seeding of cells

- Seed 2 ml of a cell suspension containing 200 000 cells/ml in a 35 mm cell culture dish (Nunc, Nunclon™ Surface or equivalent).
- Incubate cells for 24 hours in the incubator (37°C, 5% CO_2).

5.3. Exposure (24 hours after seeding)

5.3.1. Chemicals (positive control substance MMS)

- Add cell medium containing the test substance, the positive control substance (positive control) or the solvent (negative control)
- Incubate the cells for the required time (37°C, 5% CO_2)
  ➔ 24 hours

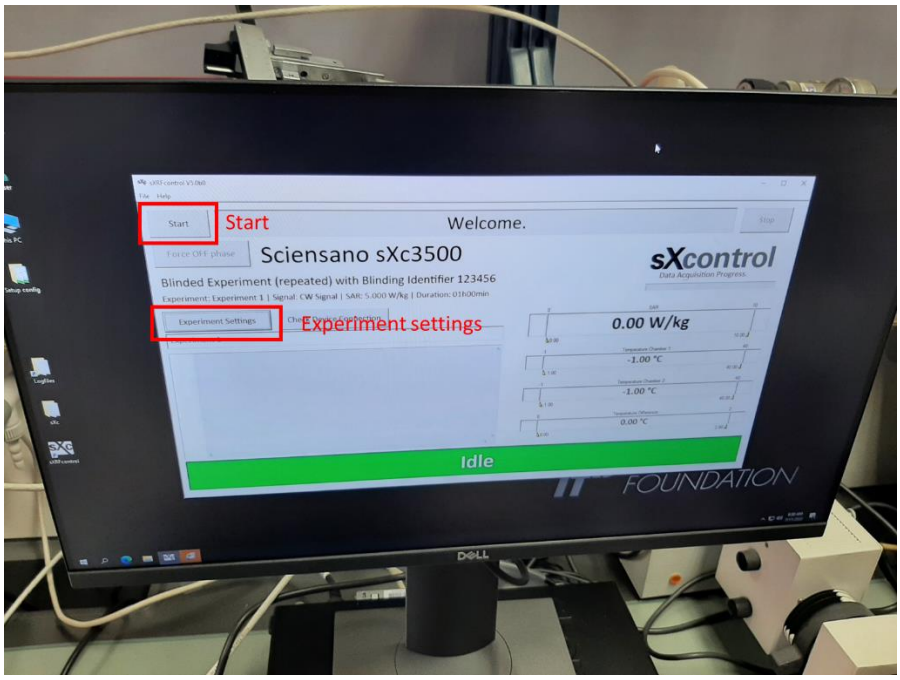
- Remove the treatment medium.
- Rinse with PBS (only for the experiments with S9).
- Add 2 ml of cytoB working solution (3 µg/ml) to each well.
- Incubate for 24 hours (37°C, 5% CO_2).

5.3.2. RF-EMF

- Add cell medium containing the positive control substance (MMS, positive control) or the solvent (negative control).
  Remark:

1. 4 dishes should always be used for the negative control (2 per exposure chamber) and four for the positive control (two per exposure chamber) to ensure the correct amount of samples to achieve statistical significance. Additionally, an incubator control can be added, including additional negative and positive control samples

- Place the cells inside the 5G exposure system (inside the sample holder) and incubate the cells for the required time (37°C, 5% CO_2)



  - For a more in depth description on how to set up the exposure system, see the "sXc 3500 exposure system" guidelines
  - In the "experiment settings" tab, adjustments can be made including SAR, exposure duration and the blinding ID of the experiment
  - Pressing start will enable the 5G EMF to flow inside the exposure chambers and pressing pause will temporarily disable this. It can be enabled again by pressing "start" again.
  - Experiments should always be performed blind, this means that only one of the chambers will be exposed at the same time. This can be decoded by sending the blinding ID to the people at IT'IS (sXc@itis.swiss). This means that the number of samples (2 Negative and 2 Positive) put inside the two exposure chambers should always be identical, since it is unknown which one of the chambers will be exposed beforehand.

5.4. Cytochalasin B treatment

- After the exposure finishes, remove the treatment medium.
- Rinse with PBS.
- Add 2 ml of cytoB working solution (3 µg/ml) to each well.
- Incubate for 24 hours (37°C, 5% CO_2).

- Remove the culture medium with cytoB.
- Add 500 µl of TrypLE to each well.
- Incubate for 10-15 minutes at 37°C. Add 1000 ml of culture medium to each well to block the trypsinisation.
- Add the cell suspension to a 15 ml conical glass centrifuge tube.
- Rinse with 1000mL culture medium to get any remaining cells and add it to the respective glass centrifuge tube.

5.5. Fixation & Slide Preparation

Remark: Prepare the KCl solution (35°C) and the ice-cold fixators 1 and 2 well in advance!

- Remove the culture medium with cytoB.
- Add 500 µl of TrypLE to each well.
- Incubate for 10-15 minutes at 37°C. Add 1000 ml of culture medium to each well to block the trypsinisation.
- Add the cell suspension to a 15 ml conical glass centrifuge tube.
- Rinse with 1000mL culture medium to get any remaining cells and add it to the respective glass centrifuge tube.
- Centrifuge 5 min at 102xg (= 700 rpm)
- Aspirate the supernatant (leave enough supernatant to homogenize the cell pellet).
- Homogenize the cell pellet by tapping (or gently vortexing)
- Slowly add 1 ml of 0.075 M KCl (at 35°C) (drop by drop) while gently vortexing cells (starting with tube nr°1).
- Leave 10 min at room temperature (no longer!!!).
- Slowly add 1 ml (freshly prepared!) ice-cold fixator 1 while gently vortexing cells (start again with tube nr°1).
- Add 1-3 drops of paraformaldehyde to each glass tube for better preservation of the cytoplasm (optional)
- Leave 10 min at room temperature.
- Centrifuge 5 min at 102xg (=700rpm).
- Aspirate the supernatant (leave enough supernatant to homogenize the cell pellet).
- Gently homogenize the cell pellet by vortexing lightly.
- Gently add 1 ml of ice cold fixator 1 in drops by using a pasteur pipette
- Gently add 4 ml of ice-cold fixator 1.
- Centrifuge during 5 min at 102xg (=700rpm).
- Aspirate the supernatant (leave enough supernatant to homogenize the cell pellet).
- Homogenize the cell pellet by tapping (not vortexing).
- Gently add 4 ml of ice-cold fixator 2.

- Centrifuge during 5 min at 102xg (=700rpm).
- Aspirate the supernatant but leave enough fixator (+- 1mL) to obtain an adequate number of cells in suspension for cell spreading.
- Homogenize the cell pellet (gently vortexing).
- Add 2-3 drops of the cell suspension on dry, pre-cleaned slides. Make sure cells are equally spread throughout the slide. Prepare at least 5 slides for each condition (to have +- 5000 cells to analyse).
- Mark the slides with pencil. The following parameters must be marked on the slide:

  Dosimetry and position in the waveguide, absence or presence of positive control subtance and slide number

  Optional: Date, the study number, the experiment number

- Dry slides overnight.

5.6. DAPI staining for MN scoring

- Add 2 drops of Vectashield mounting medium containing DAPI on each slide.
- Put cover (24x50 mm).
- Leave for at least 15 min before starting microscopic analysis of micronuclei.

5.7. Propidium/DAPI staining for CBPI analysis (one slide per condition)

- Pipette 100 µl of the Propidium Iodide working solution on each slide
- Put cover (24x50)
- Leave for 10 minutes in the dark
- Remove cover and rinse with RO water in a 50 ml reservoir
- Rinse a second time with RO water in another 50 ml reservoir
- Air Dry slides for at least 1 hour in the dark
- Add two drops of Vectashield mounting medium containing DAPI on each slide
- Put cover (24x50)
- Leave at least 15 minutes before starting microscopic CBPI analysis (see 3.7.4)

Remarks:

1) Staining is done in the laminar flow
2) If DAPI stained slides are used for PI staining, remove coverslip after microscopic analysis of slides for detection of micronuclei and wash briefly with RO water.

6. Microscopic analysis of slides – detection of micronuclei in binucleated cells

Remark: Switch on the fluorescent lamp at least 15 minutes before analysis.

IMPORTANT: Life span of lamp is limited (300-400 hours).

Preferably use the microscope (lamp) for a continuous longer period instead of multiple smaller periods. Make sure the lamp is switched off after automatic analysis (choose "Shut Down" at "search end" or "continue" if visual analysis of MN is performed immediately).

6.1. Identification of the slides (SET UP)

- Fix slides in a frame:
- Max 8 slides in one frame (see figure 1). The slide in the right hand position is slide No1.
- Up to 10 frames can be used (80 slides).
- If more than 1 frame is analyzed, frames need to be placed in the slide-feeder (frame NR 1 in position 1 etc….).

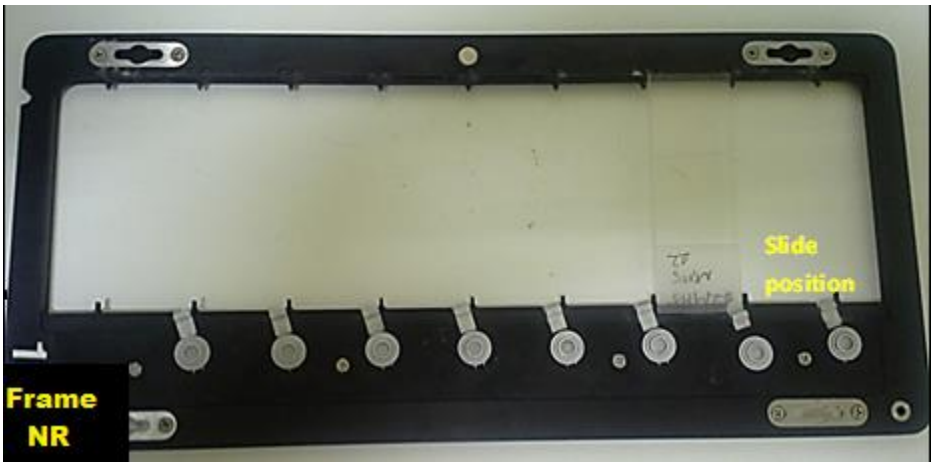
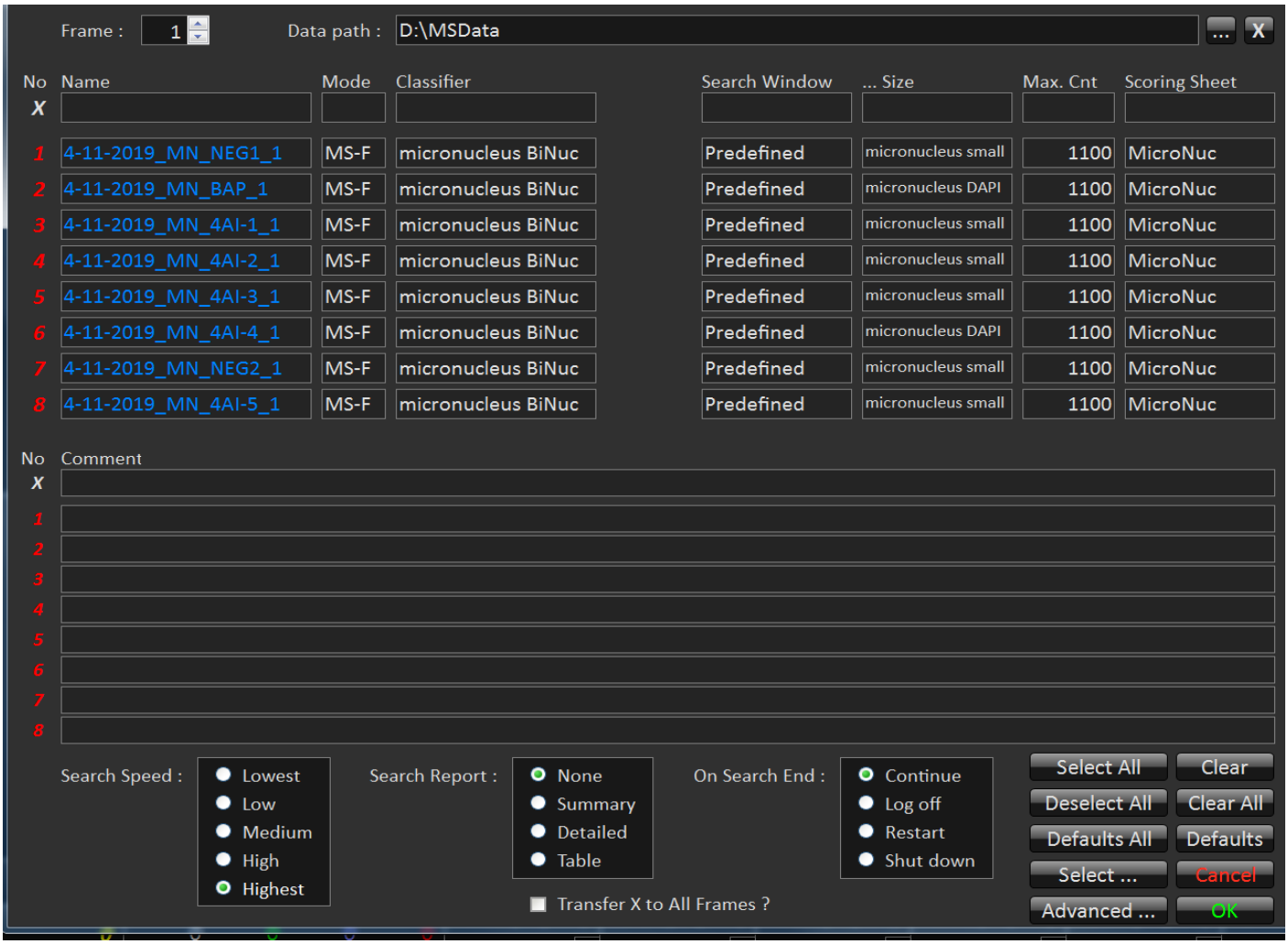


Figure 1: Slide holder

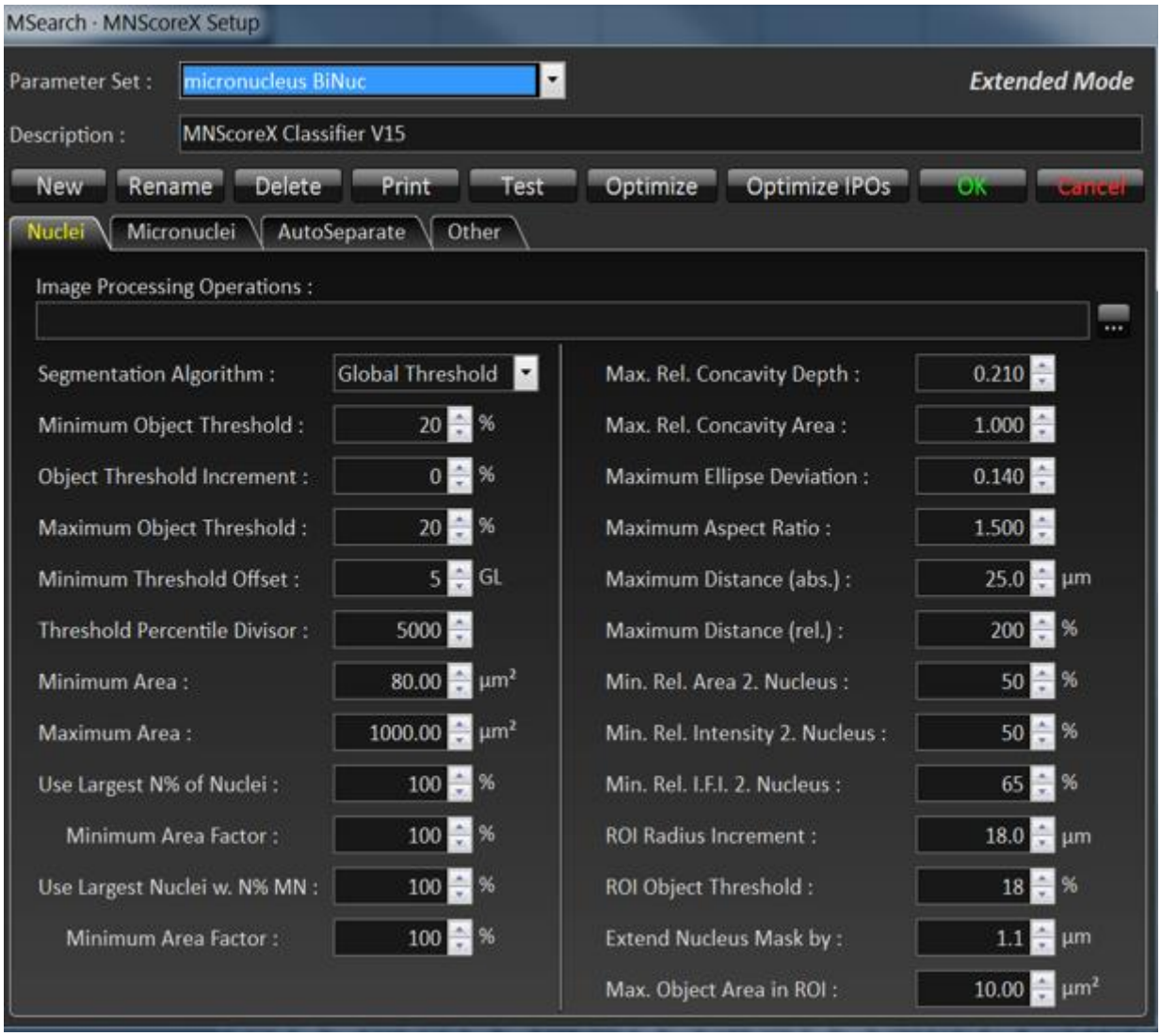
- Open computer
- Open METAFER4 software (double click METAFER4 icon on desktop)
- Open SET UP page
- Choose:
  1) Frame number (1 to 10) (if frame 1 is used also frame 1 should be selected in the SET UP menu)

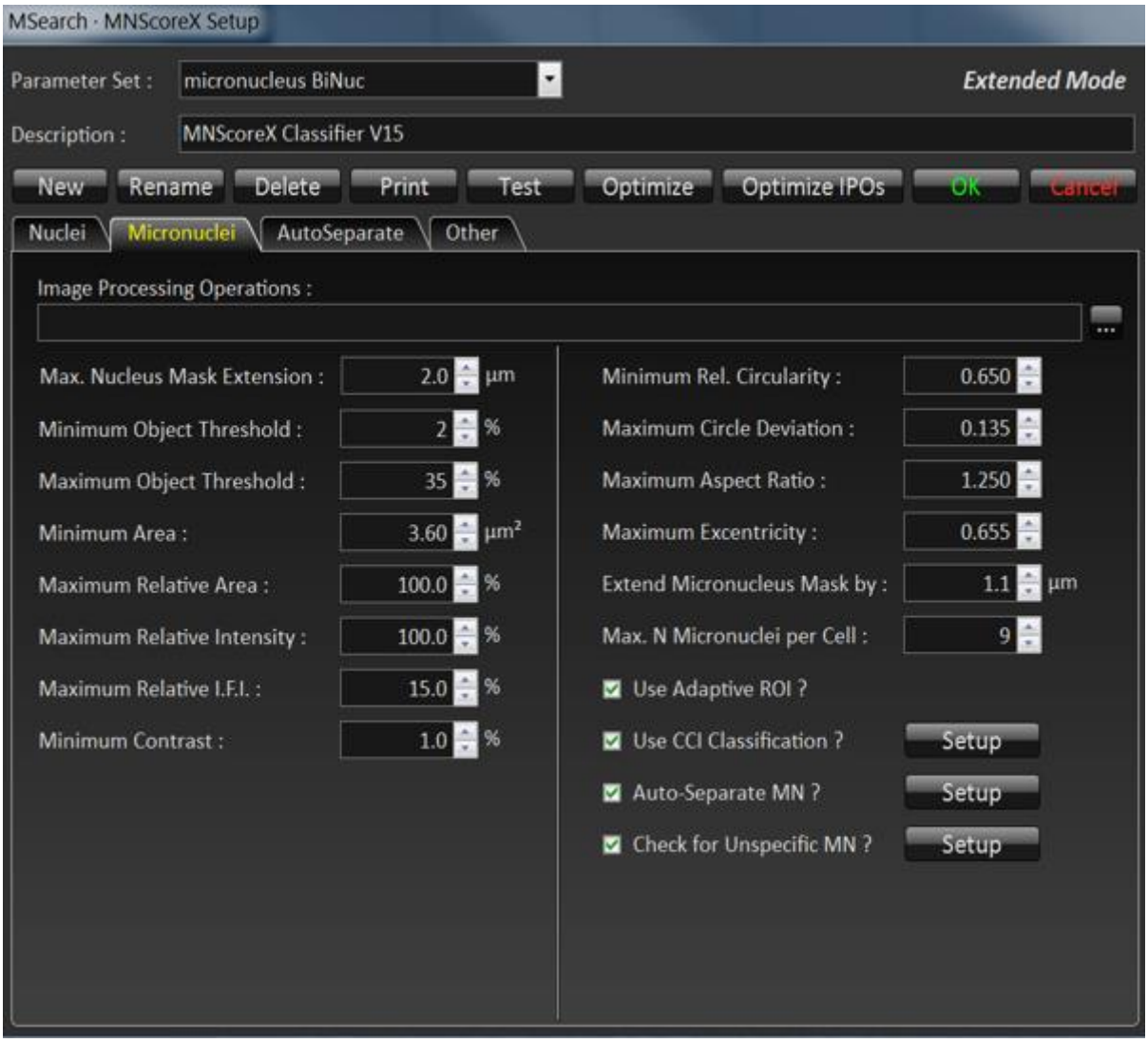
2) Highlight slides that need to be analysed (1 to 8) in red
3) Identify the slides as follows:
   XX-YY-ZZZZ_MN_A_B
   X = day / Y = month / Z = year / A = sample name/ B = slide number
4) Mode MS-F (MSearchFL)
5) Classifier: micronucleus Bi-Nuc
6) Search Window = Predefined
7) …Size
   Micronucleus DAPI or Micronucleus small (smaller window)
8) Max Count
   10000
9) Scoring Sheet: MicrNuc
10) Search Speed: Highest
11) Search Report: None
12) On Search End: Continue OR Shut Down (for an overnight analysis)

- Click OK



An overview of the parameters of the classifier micronucleus-BiNuc is provided below. These parameters are automatically applied and should not be further adjusted.


MSearch - MNScoreX Setup
Parameter Set :
micronucleus BiNuc
Extended Mode
Description :
MNScoreX Classifier V15
New
Rename
Delete
Print
Test
Optimize
Optimize IPOs
OK
Cancel
Nuclei
Micronuclei
AutoSeparate
Other
Image Processing Operations :
Segmentation Algorithm :
Global Threshold
Minimum Object Threshold :
20
%
Object Threshold Increment :
0
%
Maximum Object Threshold :
20
%
Minimum Threshold Offset :
5
GL
Threshold Percentile Divisor :
5000
Minimum Area :
80.00
µm²
Maximum Area :
1000.00
µm²
Use Largest N% of Nuclei :
100
%
Minimum Area Factor :
100
%
Use Largest Nuclei w. N% MN :
100
%
Minimum Area Factor :
100
%
Max. Rel. Concavity Depth :
0.210
Max. Rel. Concavity Area :
1.000
Maximum Ellipse Deviation :
0.140
Maximum Aspect Ratio :
1.500
Maximum Distance (abs.) :
25.0
µm
Maximum Distance (rel.) :
200
%
Min. Rel. Area 2. Nucleus :
50
%
Min. Rel. Intensity 2. Nucleus :
50
%
Min. Rel. I.F.I. 2. Nucleus :
65
%
ROI Radius Increment :
18.0
µm
ROI Object Threshold :
18
%
Extend Nucleus Mask by :
1.1
µm
Max. Object Area in ROI :
10.00
µm²


MSearch · MNScoreX Setup
Parameter Set :
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Extended Mode
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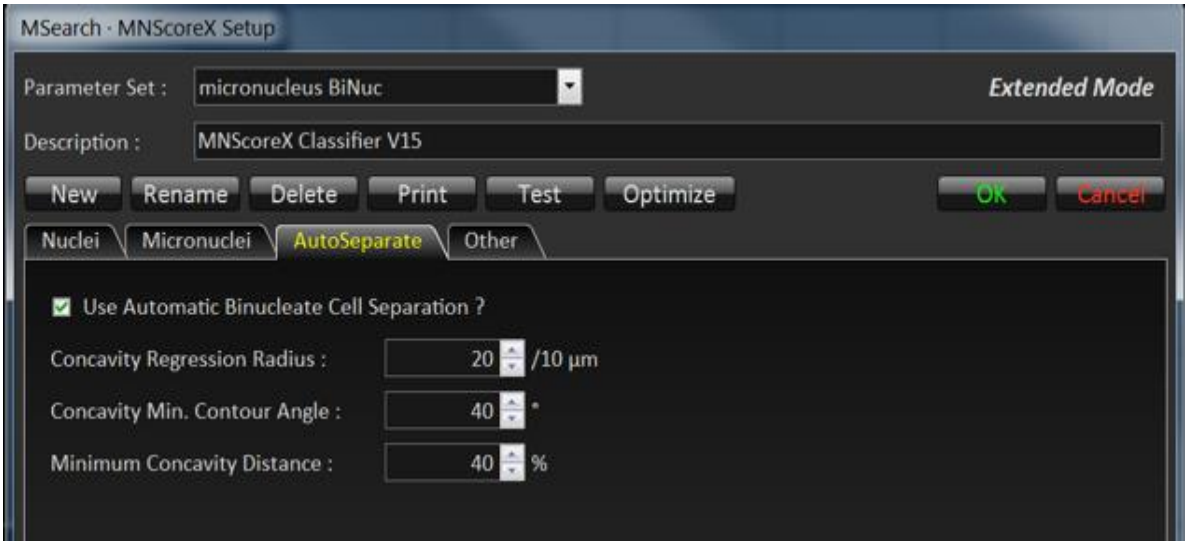
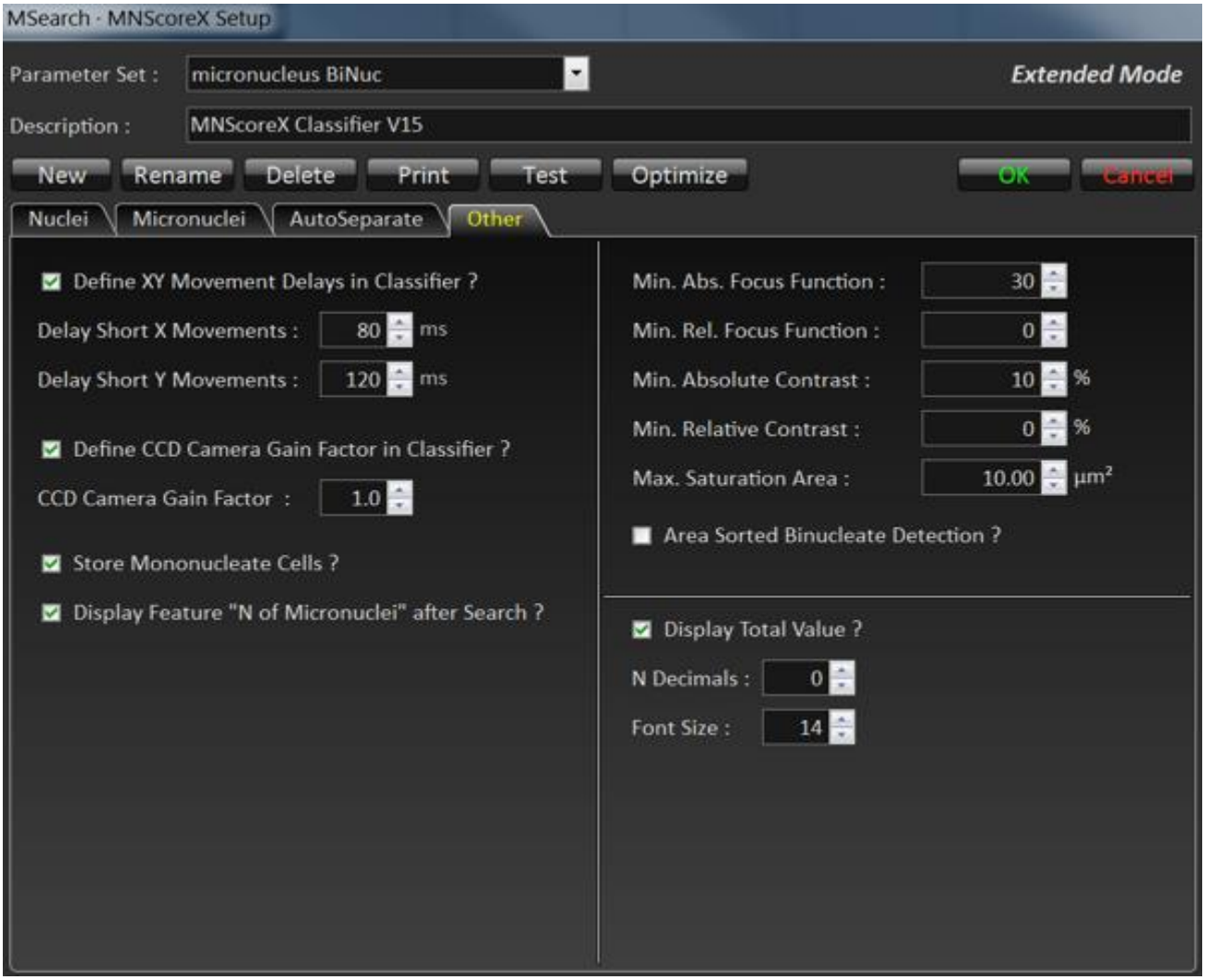
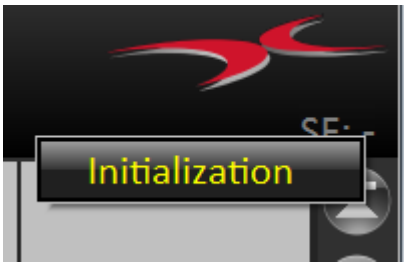
MNScoreX Classifier V15
New
Rename
Delete
Print
Test
Optimize
Optimize IPOs
OK
Cancel
Nuclei
Micronuclei
AutoSeparate
Other
Image Processing Operations :
Max. Nucleus Mask Extension :
2.0
µm
Minimum Object Threshold :
2
%
Maximum Object Threshold :
35
%
Minimum Area :
3.60
µm²
Maximum Relative Area :
100.0
%
Maximum Relative Intensity :
100.0
%
Maximum Relative I.F.I. :
15.0
%
Minimum Contrast :
1.0
%
Minimum Rel. Circularity :
0.650
Maximum Circle Deviation :
0.135
Maximum Aspect Ratio :
1.250
Maximum Excentricity :
0.655
Extend Micronucleus Mask by :
1.1
µm
Max. N Micronuclei per Cell :
9
Use Adaptive ROI ?
Use CCI Classification ?
Setup
Auto-Separate MN ?
Setup
Check for Unspecific MN ?
Setup


MSearch · MNScoreX Setup
Parameter Set :
micronucleus BiNuc
Extended Mode
Description :
MNScoreX Classifier V15
New
Rename
Delete
Print
Test
Optimize
OK
Cancel
Nuclei
Micronuclei
AutoSeparate
Other
Use Automatic Binucleate Cell Separation ?
Concavity Regression Radius :
20
/10 µm
Concavity Min. Contour Angle :
40
°
Minimum Concavity Distance :
40
%


MSearch · MNScoreX Setup
Parameter Set :
micronucleus BiNuc
Extended Mode
Description :
MNScoreX Classifier V15
New
Rename
Delete
Print
Test
Optimize
OK
Cancel
Nuclei
Micronuclei
AutoSeparate
Other
Define XY Movement Delays in Classifier ?
Delay Short X Movements :
80
ms
Delay Short Y Movements :
120
ms
Define CCD Camera Gain Factor in Classifier ?
CCD Camera Gain Factor :
1.0
Store Mononucleate Cells ?
Display Feature "N of Micronuclei" after Search ?
Min. Abs. Focus Function :
30
Min. Rel. Focus Function :
0
Min. Absolute Contrast :
10
%
Min. Relative Contrast :
0
%
Max. Saturation Area :
10.00
µm²
Area Sorted Binucleate Detection ?
Display Total Value ?
N Decimals :
0
Font Size :
14

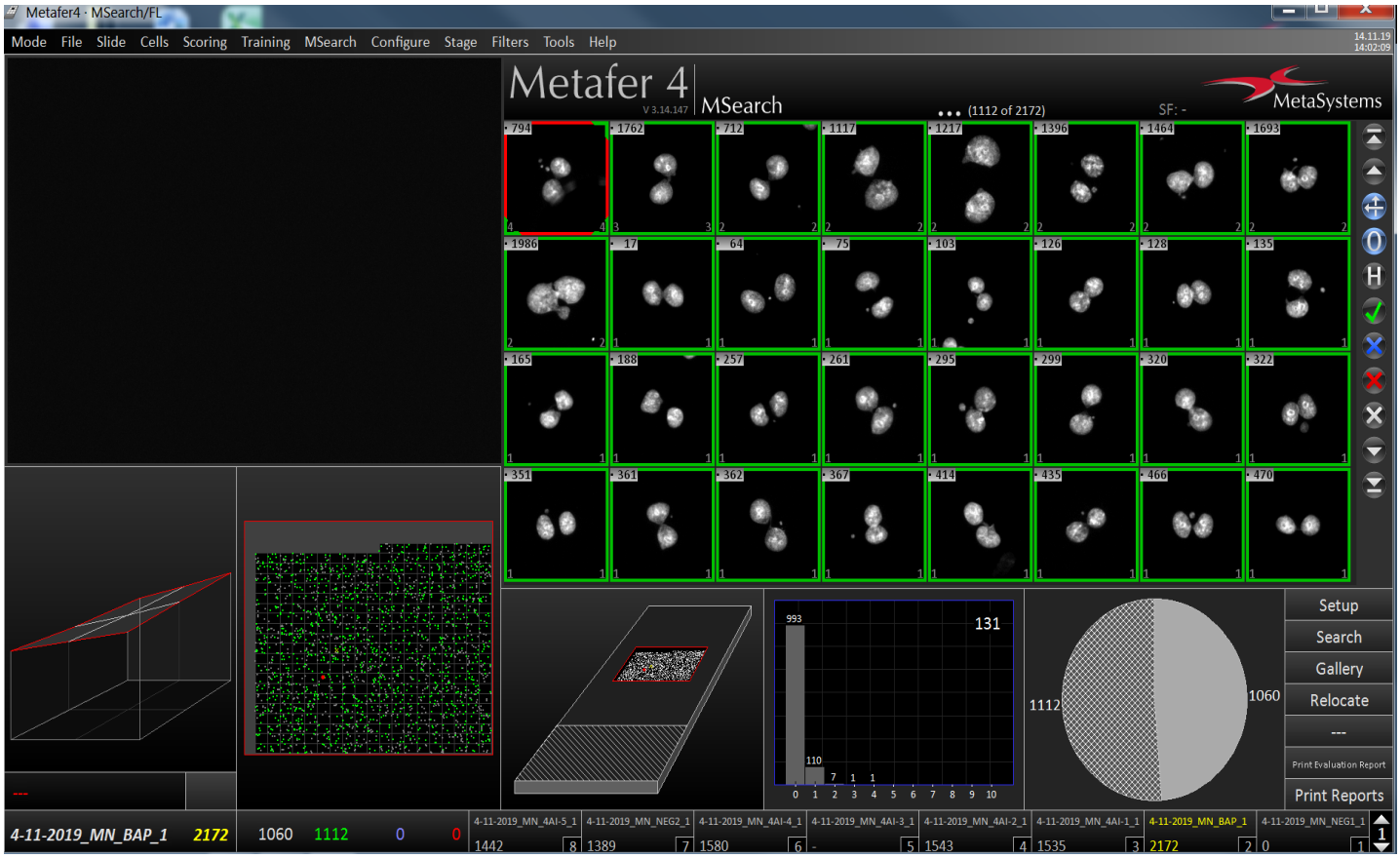
6.2. Analysis (SEARCH)

Remark: If the slide feeder is used, right mouse click on the SF icon to initialize the slide feeder (= initialization).



When only one frame is analysed, put this frame immediately on the slide holder of the microscope (no need to use the slide feeder).

- Click on SEARCH. The first slide from the identified slides in the SET UP menu is automatically selected.
- Focus microscope on a field that contains stained nuclei of binucleated cells representative for the slide and free from unusually bright artifacts or impurities.
- Make sure the camera is open for screen view (screen view/microscope view switch).
- Click OK.
- Automated analysis of all identified slides is performed. Preset number of images (usually 1000 binucleated cells/slide) will be automatically captured per slide and stored into a gallery. For each condition, at least 2 slides are scored in order to obtain min 2000 binucleated cells.
- In the event of an equivocal result, analysis may be extended up to 5000 binucleated cells.
- Mono-nucleated cells from the same field are captured and stored at the same time. Ratio mono/bi nucleated is presented in a pie chart
- Binucleated cells with 0,1,2,3…10 micronuclei are presented in a bar graph



6.3. Visual control of captured MN

After automated analysis, the detected micronuclei are analyzed visually (choose manually Objective 40x and DAPI filter on the microscope) in order to evaluate whether they fulfill the following criteria:

1) Micronuclei are morphologically identical to the main nuclei but they are smaller The diameter of micronuclei varies between 1/16 and 1/3 of the mean diameter of the main nuclei
2) Micronuclei are not linked or connected to the main nuclei.
3) Micronuclei usually have the same staining intensity as the main nuclei but occasionally staining may be more intense (with very small micronuclei) or paler (with larger micronuclei).
4) Micronuclei may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.

remark:
leave slides in the slide holder for visual check! When clicking on a cell from the gallery, the cell will be displayed in the middle of the field visible through the microscope.

6.4. Evaluation of the Cytokinesis-Block Proliferation Index (CBPI)

The Cytokinesis-Block Proliferation Index (CBPI) is determined in order to evaluate cytotoxicity based on the number of mononucleate, binucleate and multinucleate cells.

- Choose manually Objective 20x and DAPI/FITC/Texas Red filter on the microscope.
- Count manually the number of mononucleate, binucleate and multinucleate cells on the acridine orange stained slides (total of 500 cells).
- Calculate CPBI by applying the following formula:

$$CPBI = (M1 + 2M2 + 3Mn)/N$$

with

M1 = number of mononucleated cells

M2 = number of binucleated cells

Mn = number of multinucleated cells

N = total number of cells scored

7. Evaluation of the results

7.1. Study report

For each *in vitro* micronucleus experiment a new MN template (Study Report) should be filled in.

This template contains:

1) Study parameters
2) Exposure Information
3) Results & statistics – one tab for each test item

Remark:

1) Statistical analysis using a Fisher's exact test and chi-square test is separately performed in GraphPad
2) Only the most recent Template should be used.
   \\sciensano.be\fs\1442_ExpToxi_Employee\L1 Calibration Material & Products\kwaliteit labo\Templates Labo\MN

7.2. Historical Data

Negative and positive control values should be added to the historical data file in order to build historical control ranges.

\\sciensano.be\fs\1442_ExpToxi_Employee\L1 Calibration Material & Products\kwaliteit labo\Historische Data negatieve & positive controles\MICRONUCLEUS

7.3. Criteria for a positive and negative result

When evaluating the results, the following criteria should be considered:

- The test is regarded as clearly negative if there is no increase of either statistical or biological significance in the number of micronuclei at any of the concentrations tested compared with concurrent vehicle controls.
- The test is regarded as clearly positive if there is an increase in the number of micronuclei that is of statistical and biological significance and that clearly demonstrates a concentration dependent trend.
- If an increase does not fulfill all criteria for a positive result or in case of an equivocal result scoring additional cells (where appropriate) or performing a repeat experiment possibly using modified experimental conditions (e.g. concentration spacing) could be useful. Importantly, biological relevance has to remain the primary consideration.
- In rare cases, even after further investigations, the data set will not allow a conclusion of positive or negative, and will therefore be concluded as equivocal

8. Norms and references

- Fenech M. 2007. Cytokinesis-block micronucleus cytome assay. Nat Protoc. 2(5):1084-104.
- OECD. 2014. OECD TG487: In vitro Mammalian Cell Micronucleus Test
- Doherty et al. 2016. The in vitro micronucleus assay. In Genetic toxicology testing : a laboratory manual.