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Micronucleus cytome assay in buccal cells – Method development

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Abstract

Objectives
Our aim is to develop a buccal cell micronucleus (MN) assay relying on automated image analysis, with the MetaSystems Metafer image cytometry system. A prerequisite for this objective is a well-optimized slide preparation protocol. Our objectives were to: evaluate samples stored for 5, 10 and 10 days at temperatures: room temperature (RT), 4°, and -20°C. Moreover, we wanted to assess effectiveness of self-collection by participants using the number of cells harvested as determinant, and finally, test a particular DNA stain, known as DAPI, in the automated system.

Method
After ethics committee approval, we recruited 36 healthy participants aged 19 to 30. Buccal cell collection with a cytobrush was performed by participants themselves (self-collection), following given written instructions. The cells were extracted from the cytobrushes by gently shaking them in Saccomanno's solution (10ml). This solution was split into two identical filled (5ml) tubes. One tube was used for direct slide preparation (control) and the second was stored for 5, 10 or 20 days either at -20°**,** 4° or RT before processing. Slides were prepared following a specific and adapted protocol, DAPI stain was applied, and slides were reviewed by fluorescence microscope. During microscope analysis, each slide was cautiously examined by the researcher, and two images were taken at x2.52 magnification at distinct regions. Cell counting was performed on both images and we regarded the sum for comparison between slide cell quantities. Each experimental value was paired with their respective control by computing experimental value/control value ratios (Exp/Ctrl). Each slide was subjectively given a score by the same researcher depending on sampling quality from their associated participant: 1 for poor quality; 2 mediocre quality; and 3 for good quality.

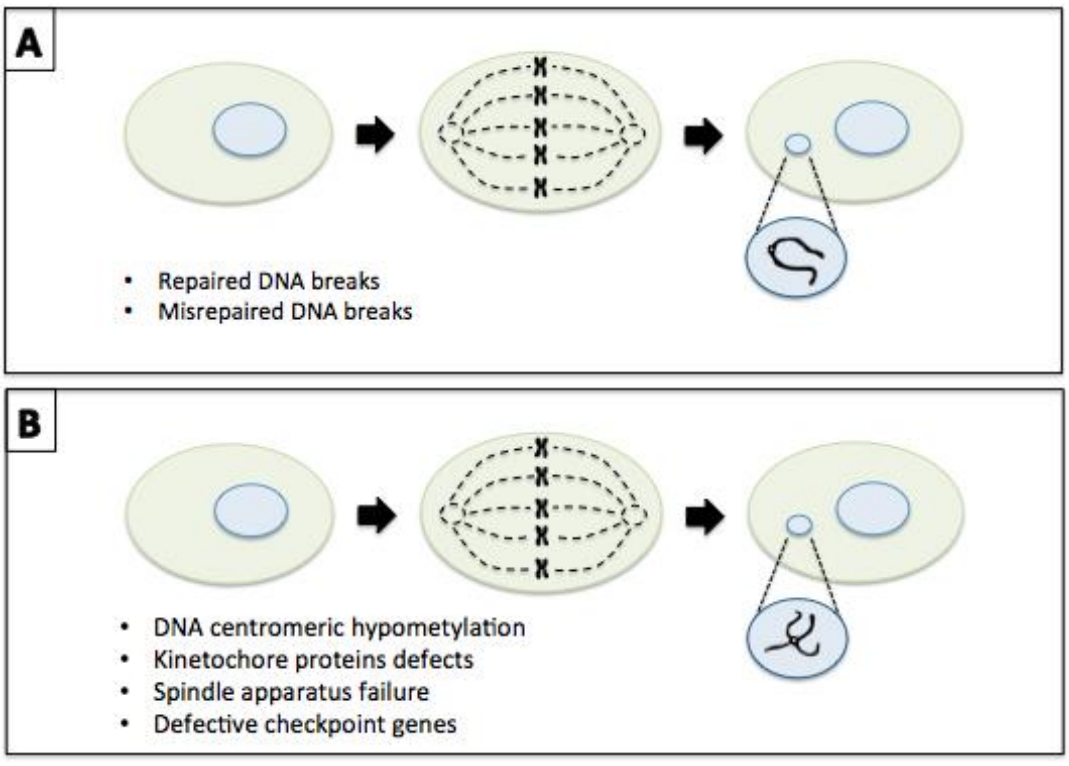
Results
In total, 12,240 cells were counted, with a mean value of 170 cells per slide. At 5 and 10 days, independent of storage temperature, mean ratios were all greater than 100%. This tendency was reversed after 15 days. Indeed, 10 out of the 12 stored samples (83%) had lower cell counts than their respective controls. Participants with the lowest score had a sample mean of 31 cells versus 185 and 246 from those who received 2 and 3, respectively. Finally, male participants had greater cell pools than females with a mean of 180 versus 123 cells.

Discussion
These results suggest that cells can be stored at room temperature up to 10 days. Consequently, slide quality would not be affected by temperature if participants collected their samples at home and directly sent them by secure post. However, results discourage cell storage for more than 10 days, specifically at room temperature, for which an important decrease of cell count was observed. Cell number was correlated with sampling quality by the participants: when swabbing was poor, slides had a lower cell count, less than half of the total average. DAPI staining and overall slide quality were satisfactory. Nevertheless, cytoplasm staining is sometimes poorly defined in the microscope, which makes MN identification harder. It can be difficult to differentiate MNi from artifacts such as staining particles or debris.

1. Introduction

In current society, humankind is more and more subject to various chemical and physical genotoxins, which can induce DNA damage. However, it is well known that genome damage is at the root of many degenerative and acquired diseases such as cancer. For this reason, great efforts are made worldwide to measure the impact of environmental, life-style and occupational factors on genomic integrity.

Historically, micronuclei (MNi) were first discovered at the end of the nineteenth century in red blood cell precursors by William Howell and Justin Jolly (1). Micronuclei are extra-nuclear bodies that contain whole chromosomes or fragments of a chromosome that were not included into the main daughter nucleus during cell division (2). These displaced DNA fragments are then eventually enclosed in a nuclear membrane, so that they are morphologically identical to nuclei except for their smaller size and lesser DNA content (hence the term "micronucleus" or "MN") (2). MNi are formed during mitosis, in the metaphase/anaphase transition, from acentric chromatid or chromosome fragments (3–5). Acentric chromatid fragments usually come from unrepaired or misrepaired DNA breaks, which result in asymmetrical DNA exchange during anaphase. However, whole acentric chromosomes originate from multiple processes such as DNA centromeric hypomethylation defects in the cell cycle control system or spindle apparatus failure (4–6) [Figure 1].



Mechanisms of MN formation
Acentric chromatid fragments come from unrepaired or misrepaired DNA breaks. Whole acentric chromosomes originate from DNA centromeric hypomethylation defects in the cell cycle control system, spindle apparatus failure, defective checkpoint genes or kinetochore protein defects.

Figure 1

In summary, MNi constitute an important biomarker of DNA damage. Their use for biomonitoring was first described in 1973 when studies of micronuclei formation in mammals' bone marrow exposed to various mutagens such as irradiation came to the surface (7–9).

To this day, many studies use MNi scorings to determine DNA damage. There is indeed strong evidence linking their frequency to genotoxic agent exposure, both in vivo and in vitro, in mammal and human cell lines (10–13). Moreover, numerous papers have suggested a strong relationship between cancer and MNi amplitude. In 2007, *Bonassi et al.,* found an association between the number of micronuclei in peripheral blood lymphocytes and the risk of cancer in various sites of the body. A particularly strong link was shown with stomach and intestinal cancers (14). Similar results were found in BRCA1/2+ healthy women as well as in patients with lung or pleural cancers (15–17). Furthermore, MN assays have also been used in a large range of illnesses, such as sickle cell or neurodegenerative disease, and both have been correlated with a higher MN frequency (4,18). Most of these MN assays were achieved in lymphocytes. Indeed, since 1986, a particular technique of biomonitoring in human lymphocytes, the cytokinesis-block micronucleus method (CBMN), is commonly realized (19). However, similar assays are also being used in epithelial tissues, including buccal cells, which are easily harvested from the inner cheeks of the mouth.

Buccal cells represent the first barrier against inhaled or ingested toxins and therefore constitute an important target for genomic damage. Moreover, 90% of human cancers arise from epithelial cells (20,21). Consequently, as with lymphocytes, recent research is focusing on establishing correlations between buccal MN amplitude and various toxins, cancers, lifestyle habits or radiation (20,22–25). For instance, MN frequency has been linked to uterus and breast cancers and could help assess cancer risk in the upper aero-digestive tract (26,27). Nevertheless, current literature is still too limited to confirm any correlation, hence the role of buccal MN assays in biomonitoring. To fill this knowledge gap, a standardized buccal MN cytome assay protocol for all the laboratories to use worldwide was created in 2009 (28).

However, most studies score MNi in about 1000 to 3000 cells, and report MNi per 1000 cells (‰). The analysis of MNi by visual microscopy is long and tiresome and thus is a source for scoring errors (22). The method is also subjective, which leads to inter-scorer variability even within the same laboratory (29). Furthermore, MN frequency in buccal cells is low (+/- 0.1%), so that a significant number of cells has to be scored to obtain enough statistical power (22). Accordingly, it seems necessary to develop an automated method of MN scoring, permitting to screen wider cell pools while avoiding inter-scorer variability. Such a technique, based on digital picture analysis of slides, has already been realized with considerate progress in lymphocytes and cell cultures, but poorly in buccal cells (30–32).

Our aim was to develop a buccal cell MN assay relying on automated image analysis, with the MetaSystems Metafer image cytometry system. A prerequisite for this objective is a well-optimized slide preparation protocol. At the IST, *Institut de Santé au Travail*, we focus on occupational exposure studies, therefore we also aspire to create a protocol based on self-sampling by the participants. This would permit the gathering of more samples, as workers could do the collecting at home and send the swabs back to our laboratory by secure post office services.

In this report, our objectives were to evaluate if samples can be stored up to 20 days, and if so, at what temperature would the samples still be usable. Indeed, this is an

important prerequisite if samples are to be shipped by post in the future. Moreover, we wanted to assess effectiveness of self-collection by participants using the number of cells harvested as determinant, and finally, test a particular DNA stain, known as DAPI, in the automated system.

2. Methodology

2.1. Subjects

We recruited 36 healthy participants aged 19 to 30. They were given a rapid questionnaire about their past and present medical history to confirm suitability for the study. Exclusion criteria were the following: general poor health; any present chronic rhinological condition; any history of a serious ear, nose and throat disorder (ENT) such as previous surgery or cancer; frequent use of NSAIDs or Aspirin; use of any type of blood thinner; any illness that predisposes to bleeding such as hemophilia.
This study was approved by the CER-VD (La Commission Cantonale d'Êthique de la Recherche sur l'Être Humain) and informed consent was obtained from each participant.

2.2. Material

Reagents :

- Tris-HCL (Merck KGaA, Darmstaadt, Germany)
- EDTA (Sigma-Aldrich Co., St. Louis, Mo, USA)
- Sodium chloride (NaCl, Sigma-Aldrich Co., St. Louis, Mo, USA)
- Acetic Acid glacial (Merck KGaA, Darmstaadt, Germany)
- Ethanol (Alcool fin 94%, without additives, Reactolab, Servion, Switzerland)
- MilliQ water (MilliQ, Millipore Corp., USA, installed on 07.02.2008)
- DAPI (120ng/ml, Abbott Molecular Inc.,Des Plaines, IL, USA) stored at -20°
- DePex mounting medium (BDH Chemicals, VWR International, Radnor, USA)

- **Preparation for 1L of Buccal cell buffer**: Dissolution of 1.6g of Tris-HCL (0.01M), 37.2g of EDTA (0.1M) and 1.2g of sodium chloride (0.02M) in 600ml of MilliQ water. pH regulation to 7.0 and MilliQ water addition until obtainment of 1L solution. Sterilization by autoclave at 121° for 30 minutes. Storage at room temperature (RT).

- **Fixing solution:** Pure Ethanol. Storage at 4°C.

Equipment:

- Chemical safety cabinet
- Centrifuge (Eppendorf 5810R) at speed 0-4000 rpm, operating at 9°-40°C
- Hand–held tissue homogenizer 15ml (Kontes Glass Co., Vineland, NJ)
- Cytobrush for cell collection (Cepilo cervical cell sampler, Deltalab S.L.U., Spain, cat. N° 440150)
- Conical 15ml polypropilene tubes
- V-shaped, yellow topped polystyrene 20ml tubes
- Filter holders 25mm (Swinex Millipore Corp. USA, cat. N°SX0002500)
- Nylon filters, 100µm pore size (Milipore Corp. USA, cat. N° NY1H02500)
- Disposable syringe 10ml (BD Discardit II)
- Needles 18G, 1.2mmx38mm
- Whatman N° 1 filter paper
- Pasteur pipettes (150mm, VWR, cat N° 612-1701)
- Coplin jars (glass, holding 10 single 76x26 slides horizontally)
- OLYMPUS IX81
- OLYMPUS DP80 color camera
- OLYMPUS cellSens software
- Microscope slides, cut edges, frosted end (76x26mm, 1mm thick)
- Coverslips 24x50mm
- Storage microscope slides boxes
- Alcool prep pads (Soft-Zellin)
- Gloves

2.3. Procedure

Adapted from the Buccal Micronucleus Cytome (BMCyt) assay (28).

Buccal cell collection:

1. Participants were informed of the following steps from a given written document in order to ensure equality of the information transmitted. Before collection, participants had to rinse their mouths with a glass of water. They were told to use one cytobrush for each cheek and firmly rotate each brush 10 times against the inside of the cheek wall in a circular motion. The two brushes were then stirred, one at the time, into the same tube filled with 10ml of Saccomanno's fixative to release the collected cells into the solution. Both brushes were then thrown away and the participants were dissmissed.

2. Each 10ml solution was split after proper mixing to obtain two identical 5ml filled tubes. One would then be used for direct slide preparation as a control and the second would be stored up to 20 days either at -20°, 4° or room temperature (RT), before processing (figure 2).

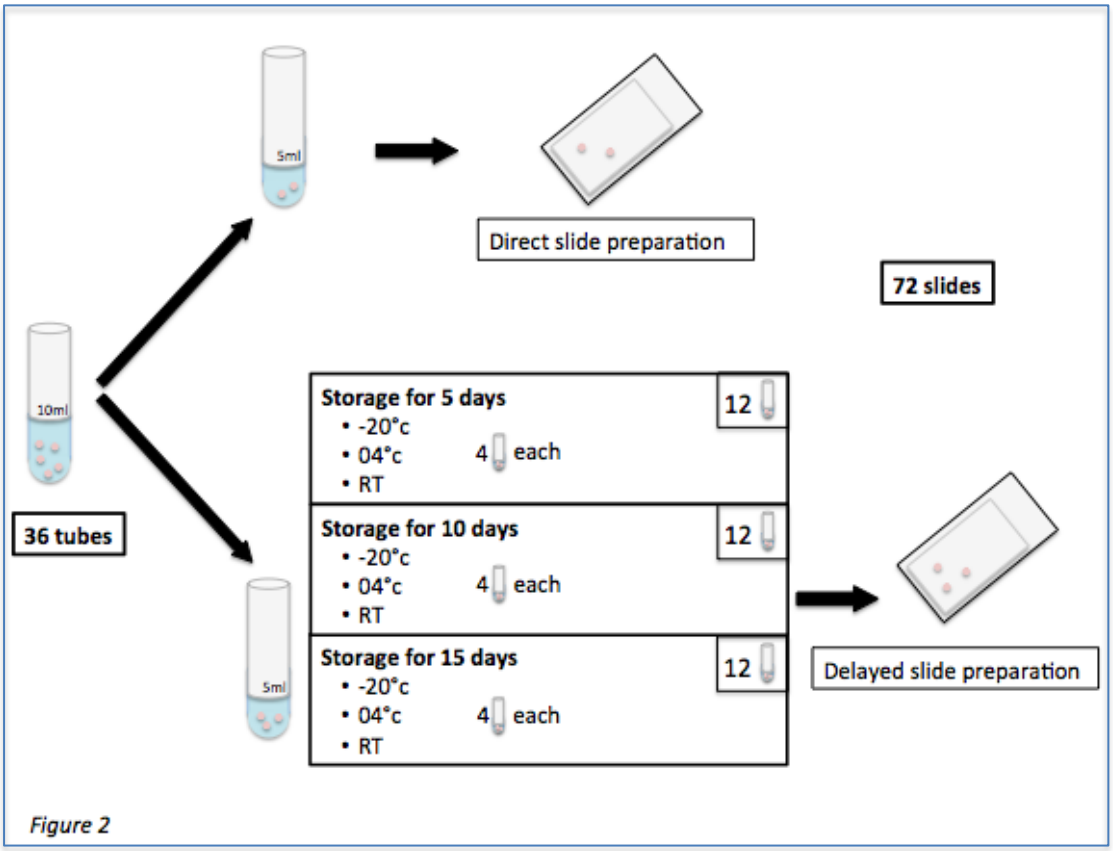


Figure 2

Slide preparation:

3. Tubes centrifugation for 10min at 1860 rpm (580g). Supernatant aspiration, leaving 1ml. Cell resuspension in 5ml of fresh buccal cell buffer using a pasteur pipette.

4. Step 2 repetition.

5. Homogenization for 2-3 minutes using the handheld homogenizer and cell transfer into 20 ml tubes.

6. Filtration trough 100µm nylon filter in a Swinex holder using a syringe with a 18G needle.

7. Tubes centrifugation for 10min at 1860 rpm (580g). Supernatant aspiration and cell resuspension in 1ml of buffer.

8. 100µl extraction by pasteur pipette and application on a slide then left to dry for at least 6 hours before staining.

Cell staining:

9. Slide immersion in a coplin jar containing 200ml of fixing solution for 30 minutes.

10. Slide immersion in coplin jars, containing 50% ETOH and 20% ETOH for 1 minute each.
11. Cell washout in a coplin jar of Milli-Q water for 2 minutes.
12. Slide drying for at least 12 hours on filter paper.
13. 40µl DAPI application on slide. Coverslip coverage applying pressure to spread DAPI on the entire surface.
14. DePex application on all the four borders of the coverglass.
15. Slide drying for at least 6 hours.
16. Slide storage at room temperature in a slidebox.

Microscopy:
Slides were examined within three days after preparation by fluorescence microscopy. All of the 72 slides were reviewed and pictures were taken at different scales (x2.52, x6.3, x12.6 and x25.2) (Figure 3). Color adjustments were used to improve image quality.

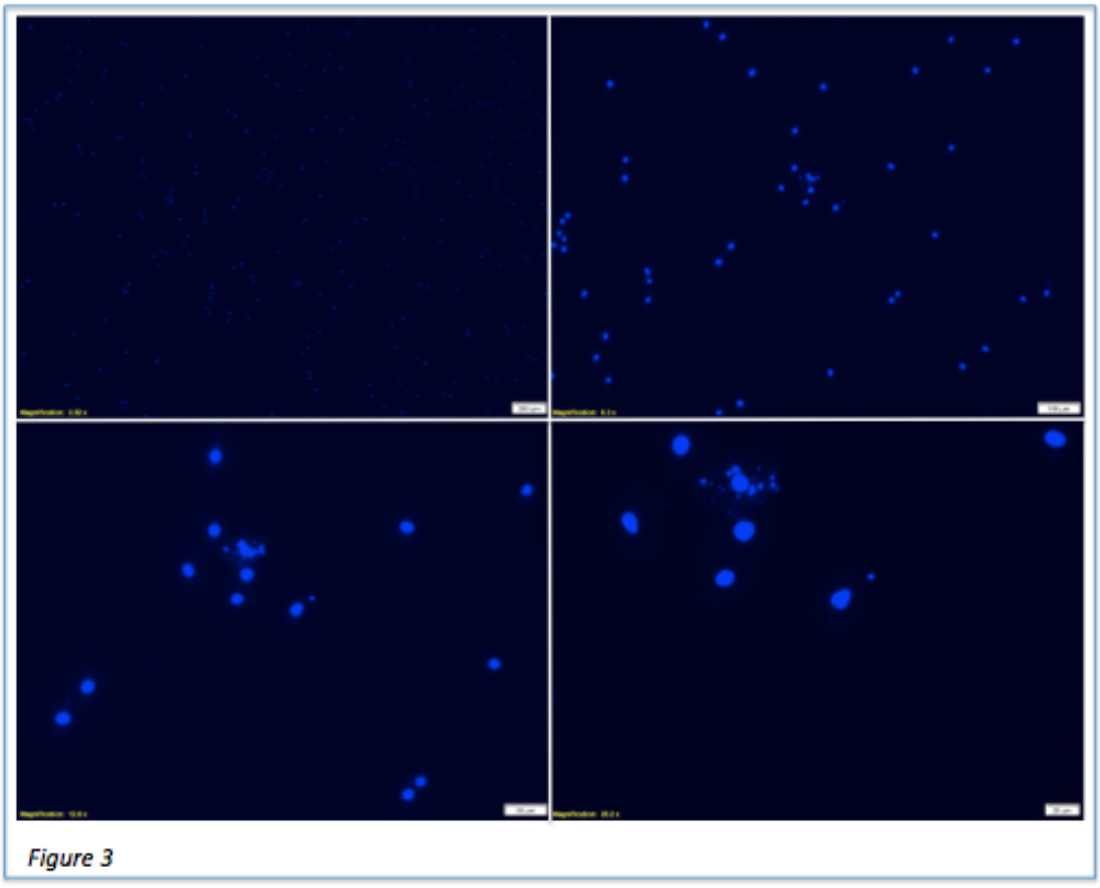


Figure 3

2.3. Variables

Entire slide cell counting was impossible due to limited access to a fluorescence microscopy. Consequently, two images were taken at x2.52 magnification on distinct slide regions, subjectively chosen for their large number of cells. Cell counting was performed on both images and their sum was used as a measure to compare the 72 slides. Counting was realized on 0.75% of the total coverslip area, which, however, contained a large sample of cells.
Each slide was also subjectively given a score by the same researcher (either 1, 2 or 3, 3 being the best) depending on the sampling quality.

2.4. Statistics

Because of the limited amount of data and the important dispersion in the outcomes, no statistical test was performed. Results are shown using charts with error bars representing standard errors (and not confidence intervals).

3. Results

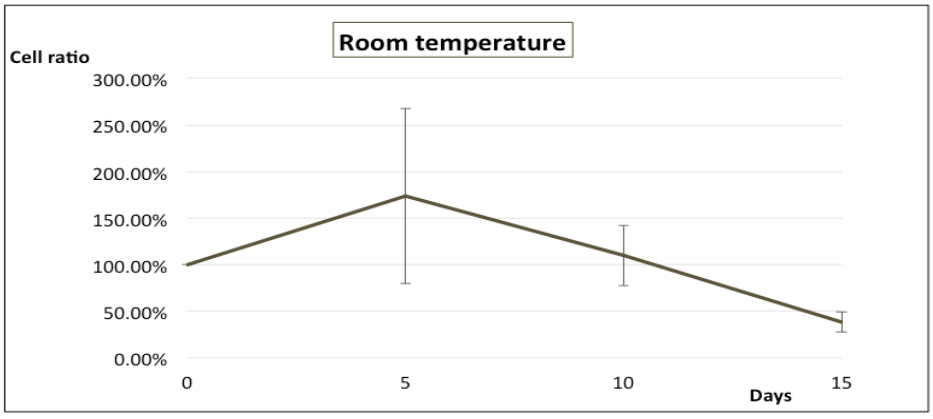
MEAN CELL NUMBER				
T° \ Days	5	10	15	Total
4	55.2	84.7	641.5	260.5
RT	275.0	86.2	145.2	168.8
-20	54.0	222.2	99.2	125.1
Total	128.0	131.0	295.3	184.8
Controls		155.2		170

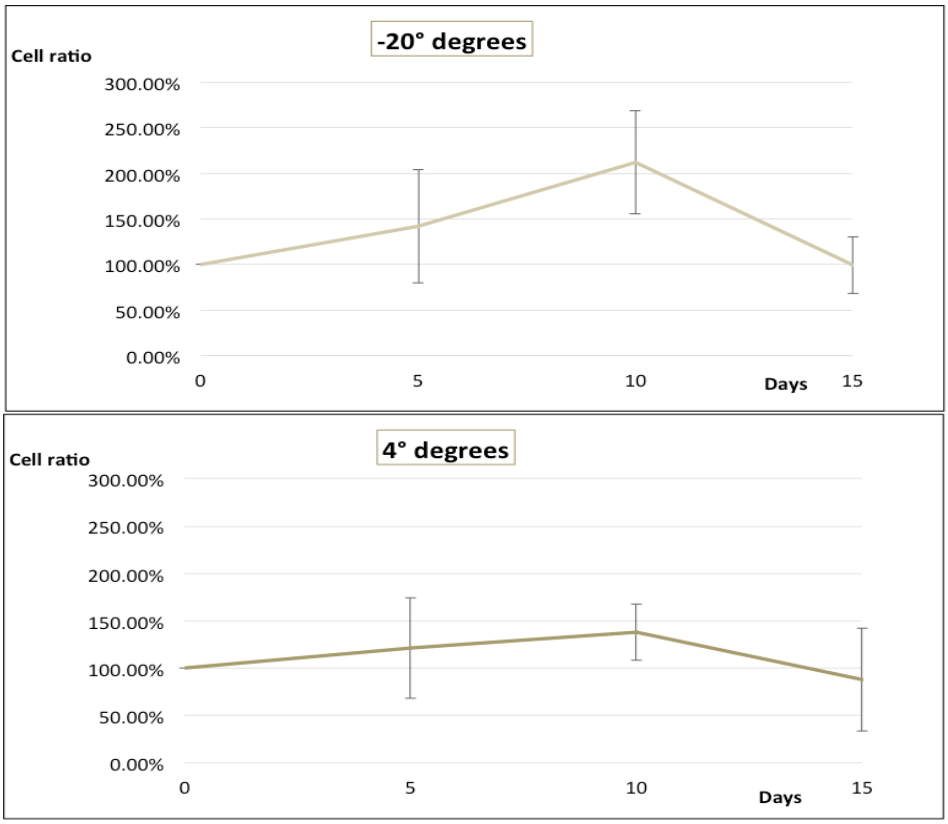
a) Average number of cells per slide according to temperature and duration of conservation, compared to controls.

Exp:Ctrl mean ratios				
T° \ Days	5	10	15	total
4	121.1%	137.9%	87.8%	115.6%
RT	173.7%	110.0%	38.4%	107.4%
-20	142.0%	212.1%	99.4%	151.2%
Total	145.6%	153.3%	75.2%	124.8%

b) Experiment:control ratios according to temperature and duration of conservation

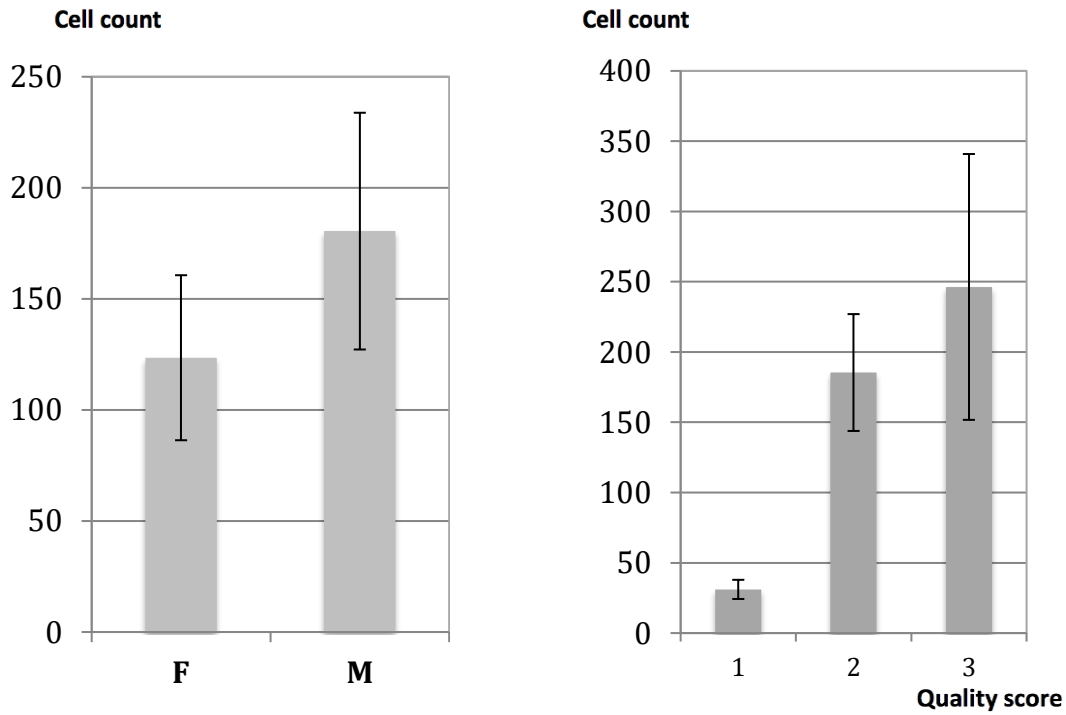
Figure 4





Experiment:control ratios according to temperature and duration of conservation

Figure 5



Average cell counts according to gender and sampling quality

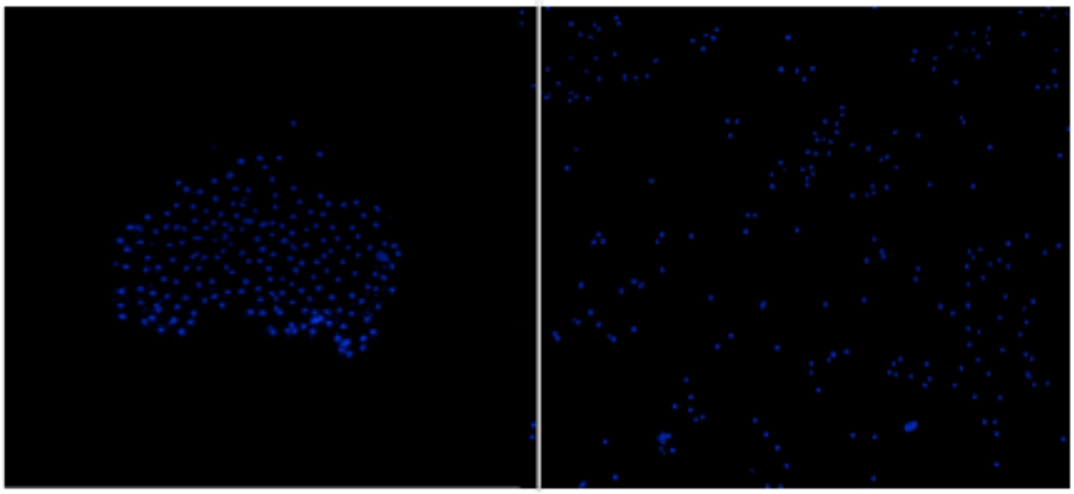
Figure 6

In total, 12240 cells were counted, with a mean of 170 cells per slide and an important dispersion. Results vary from 2 cells to 2200 cells with a standard deviation of 309 in total. A greater number of cells was counted in the experimental group than in the control sample (389 vs 202). Similarly, the mean was higher in the experimental group than in the control group (155 versus 185). Specific mean values are given in the table above (figure 4). To correctly interpret the results, each experimental value was paired with their respective control by computing experiment:control ratios (Exp/Ctrl). The outcomes are summarized in the graphs above (figure 5). Two inputs were removed as they had less than 5 cells in either one of the two categories, with consequent biased ratios. Therefore, the plots were made from 68 values, hence 34 ratios. At 5 and 10 days, independent of storage temperature, mean ratios are all higher than 100% (121%, 138% at 4°; 142%, 212% at -20°; 174%, 110% at room temperature). However, from the 24 ratios calculated, only 15 (64%) are above 1, which suggests important variability. As shown in the graphs, this tendency is reversed after 15 days. Mean ratios are all lower than 1 (88% at 4°, 99% at -20° and 38% at room temperature) and variability plays a lesser role. Indeed, 10 out of the 12 stored samples (83%) have lower cell counts than their respective controls and the 4 solutions stored at room temperature have ratio values confined between 9% and 58%.

An analysis was performed of the sample quality based on the 36 control samples, which were all subjected to the same treatment. Ten slides received the best score (3), 15 received a 2, and 11 a 1 (worst). As shown in figure 6, participants with the lowest score had a sample count of 31 cells on average versus 185 and 246 from those who received 2 and 3, respectively. Maximal cell count from these 11 participants was 70, 41% of the mean. On the other hand, cell count from participants with the highest score varies from 32 to 876 cells, a much broader range. Similarly, male participants had wider cell pools than female ones with a mean of 180 versus 123.

4. Discussion

Before starting the experience, our assumption was that cell storage in Saccomanno's solution for 5 to 10 days at 4° degrees would have no effect on cell quality or quantity. We considered it possible to observe a slight decline of the outcomes at room temperature or -20° degrees, as such conditions are usually not recommended. In the buccal Micronucleus Cytome (BMCyt) assay, it is indeed recommended to store cells at 4° degrees up to 7 days (28). Consequently, we did not expect to find an increment in cell number as we did in our results at 5 or 10 days at the three measured temperatures. This result could be a statistical fluctuation, as the sample size was small with only 34 ratios taken into account, and an important overall dispersion. Moreover, each result had an intrinsic bias: counting was performed only in a small region, which was not always representative of the whole slide. In fact, many slides had important regions of cell aggregation, while in others, cells were more uniformly spread (figure 7).
However, an alternative explanation could be that many cells were lost during the first step of slide preparation. As explained in the protocol, cells initially go through centrifugation at 1860 rpm for 10 minutes with subsequent supernatant aspiration. When solutions are stored, they genuinely separate from supernatant by gravity, which is not the case when slides are directly prepared. This extra step could result in fewer cell lost during supernatant withdrawal and therefore overall greater cell quantity.
In any case, these results suggest that cells can be stored at room temperature up to 10 days, or at least at temperatures around 17° degrees, since RT varies considerably within a year. Consequently, slide quality would not be affected by temperature if participants collected their samples at home and directly sent them by secure post.



Example of cell aggregation and comparative.

Figure 7

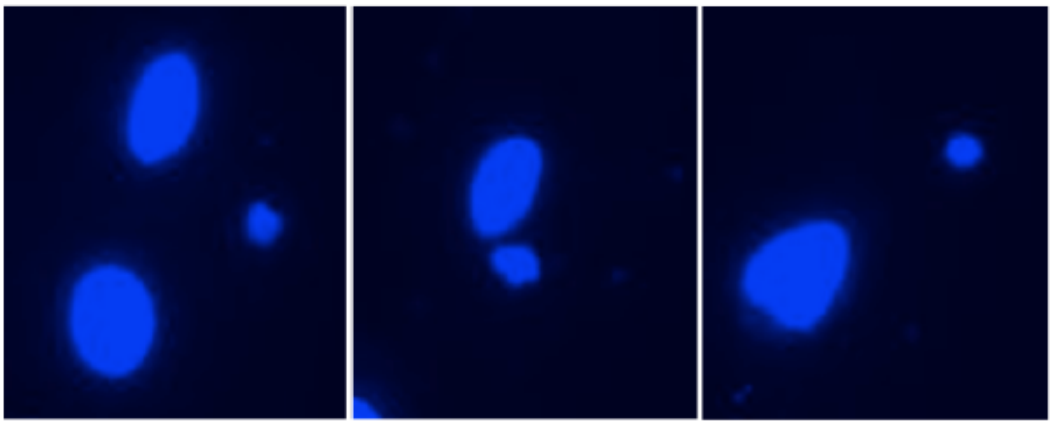
These conclusions, however, should be verified by an analysis based on a larger statistical sample. Furthermore, effects of mechanical agitation of the samples should be taken into account when considering shipping. At 15 days, mean ratios are always lower than 1, in particular at RT (38%). This suggests deterioration of cells if conserved for more than 10 days at RT, especially considering that results are more consistent, with smaller dispersion. Indeed, each of the 4 solutions stored at RT has a ratio lower than 58%. In contrast, when stored in a fridge or freezer, ratios are higher with scores of 88% and 99%, respectively. In these two cases, it his harder to draw a conclusion, as results are quite similar to the baseline (100%), but different from outcomes at 5 or 10 days. Therefore, cell deterioration could be masked by fewer cells loss during slide preparation, as mentioned before. In conclusion, it appears more appropriate to store cells only up to 10 days, as quality cannot be guaranteed afterwards.

A mean of 170 cells per slide is a small amount considering that studies usually score MNi in 1 to 3 thousand cells (22). It can be partially explained by the fact that counting was performed on a small region of the total coverslip area (0.75%) and that furthermore each sample was divided in two.
However, even within the control group, there is great variability in outcomes with scores ranging from 2 to 876 cells (2200 in the experimental group). Sampling quality by the participant plays an important role in this variability. First of all, a low sampling quality score (1) predicts a low cell counting, since none of the 11 concerned participants have cell counts above 70. In contrast, participants with the highest score (3) produce better results in average, however, 30% present with counting fewer than 70 cells. This suggests that sampling is a determinant factor in cell quantity, especially when done poorly, yet it is not the only one. In other words, poor sampling results in low cell count, but when done correctly, results still vary on account of other sources.
Indeed, it is likely that the method of slide preparation itself contributes to the observed variability, due to its numerous steps, such as centrifugation, homogenization and filtration. Even if realized with cautiousness by a single researcher, these manual procedures are still subject to intra-individual variability. For the same reason, it is important to be able to rely on good sampling to reduce variability in the outcomes. Nevertheless, it is evidently not the case here, as 11 persons, hence 31% of participants, had the lowest score.

Moreover, in this study, sampling was done in front of an examiner and participants were mostly medical students. Overall sampling quality might be lower if occupational workers, with lower medical knowledge, were to do the buccal collections at home in absence of professional witnesses. Instructions by visual aid could be a possible solution to overcome these problems. Finally, it results that men achieve better cell collection than women, as their cell average is greater by 57 cells (37%). However, this result could be purely accidental, as error bars are overlapping (Figure 6) and further data would be required to confirm or disprove such a possibility.

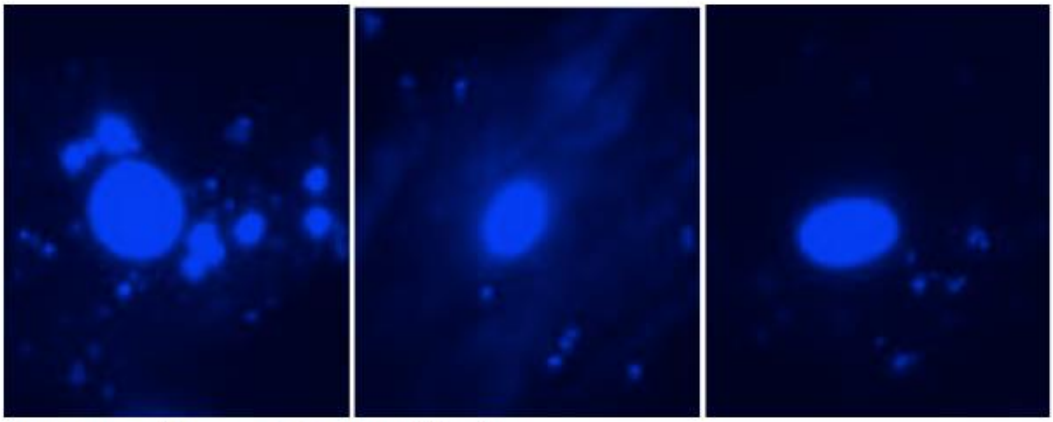
As explained in the protocol, slide preparation involves deposition of 100µl cell solution and 40µl DAPI stain before coverslip application. In total 140µl are applied on each slide, which is a relatively important quantity. Moreover, DePex is disposed on the four borders of the coverglass. Therefore, cells tend to be resting on unequal vertical positions, due to slight thickness variations within the same slide. This is not an issue when microscope focusing is done manually. Nevertheless, in automatic systems, focus is calculated from checkpoints and consequently used on the whole slide. Slight position variations are not taken into account and cells can be overlooked. To avoid such obstacle, DePex application could be removed from the protocol, as it is not essential. Indeed, DAPI tends to fade with time and slides should be analyzed rapidly after preparation in any case (33).

Even if not mentioned before, each slide was carefully examined in order to estimate if an automated MN assay is reachable. We were concerned about DAPI staining, as its usage in epithelial cells results only in a few papers (34,35). Nonetheless, staining quality was excellent with little difference between slides. DAPI is a DNA specific stain and consequently gathers mostly in nuclei; yet it also permits to slightly discriminate cytoplasm, however not systematically. This is an important issue since MNi are defined as round or oval structures contained in the cytoplasm (33,36). Therefore, when cytoplasm is not visible, MN criteria are not respected by definition. Furthermore, MNi have 3 to 16 times smaller diameters than their main nucleus, hence they can be quite limited in size. It is not always evident to single out MNi with certitude from artefacts, including debris or staining particles (figure 8,9).



Three presumable Micronuclei at 25.2x magnification. Cytoplasms are barely discernible.

Figure 8

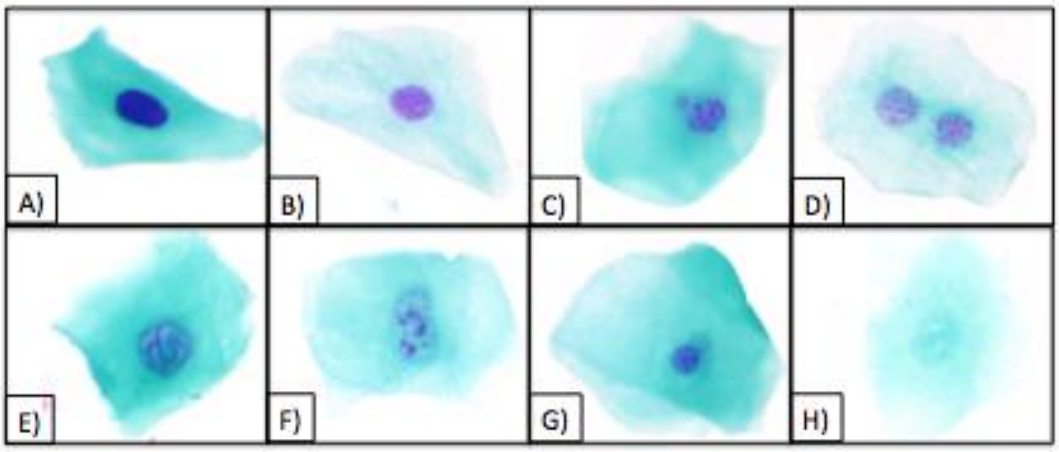


Common artifacts at 25.2x magnification.

Figure 9

Finally, compared to Feulgen and Fast Green staining (the standard method), DAPI stained cells can only be reviewed by fluorescence microscopy and not under transmitted light, which complicates their authentication (33,37). Therefore, even with a reliable automated system, capable of reviewing and selecting potential MNi, it would still be challenging for a human expert to sort them correctly.

Last but not least, in this report we only mention MNi, as our aim, at least for the moment, is to create an automated MNi slide reviewing. However, other biomarkers have been identified, and just like MNi, they are being used in lymphocytes and epithelial cells as potential screening tools. These includes biomarkers of DNA damage (nuclear buds), proliferative potential (basal cell frequency), cell death (condensed chromatin, karrhyorrhexis, pyknotic cells) and cytokinetic defects (binucleated cells) (5,28,38).



Feulgen and Light Green stain, Light microscopy.

A) Basal cell
B) Differentiated cell
C) Cell with Micronucleus
D) Binucleated cell
E) Condensed chromatin cell
F) Karyorrhectic cell
G) Pyknotic cell
H) Karyolytic cell

Figure 10

MNi scoring, but also scoring of basal and differentiated cells, as well as the above cited biomarkers (28). However, whereas these biomakers are easily detectable with Feulgen and Fast Green staining, it is rather challenging with DAPI staining even for a human eye. As an example, it is difficult to distinguish two overlapping cells from binucleated ones by fluorescence light alone. Moreover, chromatin status is barely detectable by DAPI, as generally nuclei appear homogeneously tainted. Therefore, this would be a further issue if, in the future, automated slide scoring was extended to all known biomarkers.

5. Conclusion

Our study shows that cell storage in Saccomanno's solution is practicable up to 10 days at RT, in freezer or fridge. Storage could be beneficial as it permits better cell segregatation from supernatant. However, results discourage cell storage for more than 10 days, specifically at room temperature, for which an important decrease of cell count was observed. Cell number is correlated with sampling quality by the participants: when swabbing is done poorly, slides have a lower cell count, less than half of the total average. Even with precise written indications, 31% of the participants received the lowest mark. Nonetheless, results suggest that the method of slide preparation itself has an impact on cell count, with important intra-individual variability. Finally, swabbing was performed more efficently by men compared to women, as their cell average was greater by 57 cells (37%). Results suggest that self-swabbing from participants with shipping of samples from home is achievable. However, better instructions with visual aids should be given to participants, to improve overall sampling quality. DAPI staining and slide quality were satisfactory. Nevertheless, cytoplasm staining is sometimes poorly noticeable, which makes MN identification harder. Moreover, in comparison to Feulgen and Fast Green staining, slides can only be reviewed by fluorescence; it can be difficult to differentiate MNi from artifacts such as staining particles or debris. However, due to the limited statistics available and the discussed bias in the methodology, the above conclusions should all be taken with care.

Developing a buccal cell MN assay relying on automated image analysis remains an arduous challenge. Nevertheless, with further data and optimization of the protocol, important progresses can be achieved in the future.

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