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# Suitability of Papanicolaou stain for micronuclei mass screening in buccal smears comparing reference feulgen stain and papanicolaou stain in field

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## ABSTRACT

**Background:** Micronuclei (MN) assessment so far was done directly, individually, and intra- and, here, inter-observer bias could not be eliminated. It was subjective. **Methods:** In this study, accurate and reproducible MN counting was facilitated by the use of liquid cytology and image capturing with MN identification analysis. **Results:** Comparing the two nuclear staining techniques - DNA specific Feulgen stain and commonly available Papanicolaou (PAP) stain we found that both techniques gave statistically comparable accurate and reproducible MN results. T-test and non-parametric followed by paired non-parametric and then Wilcoxon matched-pairs signed rank test tell us that both the staining procedures have no difference at $P < 0.05$. **Conclusion:** These protocols are comparable and reproducible, and may be adoptable for mass screening to assess the genotoxic effects of substances like tobacco.

**Key words:** Field collection, Image capturing, Liquid buccal cytology, Micronuclei

## Introduction

In India, 90% of all oral cancers are oral squamous cell carcinoma (OSCC). OSCC is the most common cancer in males in India. 5-year survival rate is poor 56%[1] as the patients often come in an advanced stage. Cancer is the result of mutations induced by damage in DNA. There are many mutation-inducing agents such as virus (human papillomavirus), lifestyle, nutrition deficiency, and chemicals - commonly known as carcinogens. To an extent, some substances like folic acid prevent mutations. Among various carcinogens containing substances, tobacco is one of them. Tobacco is consumed either as smoking or smokeless tobacco (SLT). SLT habit is alarmingly increasing. According to one estimate, 25% Indian are addicted to one or other form of SLT.[2] In its August 20, 2017, Deccan Herald, a newspaper from Pune, dramatized the incidence as 87% of global oral cancer in India. One sees in as young as 10 years of age and rural as well as urban areas (personal observation on the basis of increased oral submucous fibrosis (OSF) in young. Substances in tobacco are both genotoxic and cytotoxic. One sees the habit in poor working class women too.

SLT is available in many forms such as - Gutka, Kaini, Mava, and Snus. The habit is common, not only in India but also neighboring Southeast Asia countries such as Bangla Desh, Shri Lanka, Indonesia, and Thailand.[1]

Micronuclei (MN) are additional DNA material in the cell cytoplasm. Many workers have studied the frequency of cells with MN in a various subset of the population in tobacco users with or without clinical lesions and compared with normal populations.[2-5] In 1998 there was an international collaborative study on Human Micronuclei (HUMN). This was again repeated after 10 years in 2008.[6,7] 43 laboratories from 23 countries participated. One of the conclusion was in tobacco abuse MN was a good genotoxic and cytotoxic marker. HUMN study had, 43 participating, collaborative laboratories and every year there is increasing number of published paper and labs which have adopted MN studies.

Most papers quote either Tolbert *et al*.[5] or Stitch[8] for MN criteria, characterization, and methodology. For over three decades, there is no change even in methodology and no attempt to bring the same to be applied for mass application.

Tobacco abuse induces in many cases pre-cancer or potentially malignant disease (PMD). These PMD are OSF, leukoplakia, and erythroplakia.

Most studies of MN in buccal cells involve direct collection by spatula or cotton swabs from buccal mucosa, directly spreading on clean slides, stain with suitable and directly examine, and document number of MN per either 500 or 1000 epithelial cell and report. MN are identified as per criteria laid down by Tolbert *et al*.[5] or Stitch.[8] The limitations are uneven smear, overlaps, staining, subjectivity, and reproducibility. Nuclear-specific stains are expensive for a mass adaptation like PAP stain in cervical cytology. Comparative staining studies have been done.[9-11]

This presentation on methodology gives a reliable, reproducible, and with minimum bias. This is based on liquid cytology and

image capturing, documentation and analysis. Over and above the protocol in the method is adoptable for mass screening. The method compares two staining methods - DNA specific and non DNA specific, but widely used in laboratories

## Materials and Methods

### Ethics approval

This was done by Dr. Anirudh Wagh and Dr. Tanay Parikh from respective medical colleges: (1) Medical College Baroda and (2) Medical College Karamsad.

1. Institutional Ethics Committee for Human Research (IECHR) approved this study ECR/85/INST/GJ/2013 dated December 3, 2015.
2. Human Research Ethics Committee (HREC), HM Patel Center For Medical Care And Education, Karamsad approved the study.

### Selection of patients

Patients coming to outpatient clinics of teaching hospitals were asked about tobacco habit (SLT) and were randomly selected, and permission to take buccal smear was obtained. Patients with poor dental hygiene were excluded. Most patients come from rural, poor, and educationally poor background. We have consciously tried not to include smokers. There is a standard form for information on patients and submitted with samples. In recording, we adopted 10 codes as below. For this comparative study of two stains, we have included all codes.

Code 0 - no clinical lesion and no h/o tobacco usage.
Code 1–3 - it represents no clinical lesions and duration of habit. <5 years, 5–10 years, and more than 10 years
Code 4–6 - it represents clinical disease PMD. Code 4 - SMF of various grades, Code 5 - it is leukoplakia, Code 6 - it associated erythroplakia,
Code 7–9 are from clinically malignant. Code 7 - it clinically suspicious malignancy, code 8 - it clinically malignant, code-9 clinically malignant with metastatic nodes, and Code 10 is malanoplakia and other lesions.

Collection of samples from field was made as per following protocol (SOP):

1. Ask patients to rinse the mouth with water.
2. Ask patients to vigorously gargle with 2 M NaCl. Use of 2 M saline dislodges bacteria and food particles.
3. With a wet wooden spatula, inner side of buccal mucosa from both sides is gently but firmly rubbed, several times in circular motion.
4. The wetted end is dipped and shaken well in 10 ml of fixative** in a 15 ml centrifuge tube, and tube capped and identified and transported to the central lab.
5. Samples, collected as above in centrifuge tubes, are received at central lab.
6. After identification, the tubes are centrifuged for 20 min at 4000 rpm.
7. The supernatant is fully decanted.
8. The sediment is re-suspended by mixing in the same tube with whatever fluid is remaining.
9. The sediment is dropped on pre-albuminized slides. The albuminized slides are prepared by thinly spreading 1% albumin in water and kept ready for use. Albumin allows cells to be stuck to slides – 1% is optimum.
10. The drop is spread evenly in 2 cm area.
11. Do not discard sediment for 48 h.
12. Let it air dry and if needed keep slides at 45–50 c in oven (helps cell fixation with thin albumin layer on slides and drying). One can also use hot air hair dryer for quick drying.
13. Good smears show adequate number of cells (5–10 cells/HP field), minimal overlapping, and satisfactory staining.

**Fixative contains 70 ml of saline + 30 ml of Propanol + 1 ml acetic acid

A total of 47 patient samples were taken for this comparative study

### Staining

We used two staining method Feulgen and PAP for MN identification.

### Feulgen stain

1. Place slides for 30–60 min in 1 N HCl at 60 C.
2. Rinse in cold 1 HCl (optional).
3. Rinse in distilled water.
4. Place in Schiff reagent for 30–60 min.
5. Give three rinses of sulfite solution 1 min each.
6. Wash well with water.
7. Counterstain - we used aniline blue (0.5%) - others use light green.
8. Dehydrate in 70% isopropyl alcohol (propanol), then 100% isopropyl alcohol.
9. Xylene.
10. Mount in DPX and coverslip.

Note - Schiff reagent and sulfurous acid are prepared as per the standard books like Bancroft

#### Pap stain

Use standard good quality ready to use stain kit (RAPID-PAPTM). This was done according to insert of manufacture. Basic principle steps are hydration, nuclear stain, wash, dehydrate, cytoplasm stain, wash, dehydrate, xylene, DPX, and mount in coverslip.

### Acquisition of picture and data analysis

Trinocular light microscope with Magcam camera and Magvision software with picture acquisition facility was used for the study. First, use ×10 objectives to locate the appropriate field of interest and then used ×40 for the picture acquisition and with standard zigzag fashion and so as not repeat the same field. We acquired the picture and saved in the respective folder.

MN counting and MN Index determination

MN identification criteria are discussed in the discussion

Total cell counted was >1000

MN index is defined as total cells with MN/total cells counted x 100. At present, cell with more than once MN is counted as one cell only.

For counting MN one should have good smears and avoid pitfalls in MN identification. General principle is be sure and not to count doubtful MN.

## Results

MN identification and counting (this is discussed in discussion section) were done and are given in Table 1.

*T*-test and non-parametric followed by paired non-parametric and then Wilcoxon matched-pair signed rank test tells us that both the staining procedure have no difference at $P < 0.05$.

Image capture and subsequent analysis make difficult decisions easier. It makes intra- and inter-observer more reproducible less bias prone. This has not been reported in earlier studies

Figure 1a-c show advantages of image capture.

## Discussion

### How are MN formed?[12]

MN are small extranuclear DNA particles formed when chromosome fragment in dividing cells does not get incorporated in the nucleus of daughter cell. This may arise from unrepaired dsDNA breaks. That means repair pathways have become errant. Mal-segregation of the whole chromosome can occur during anaphase, and this can be due to hypomethylation of cysteine in centromeric and pre-centromeric repeat sequences. In oral mucosa, the squamous epithelium is 6–7 layers. Damages due

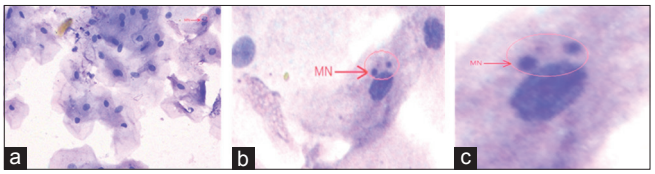


**Figure 1:** Panel (a-c) shows the image capture and enlargement for identification and confirmation of micronuclei

**Table 1: Comparative MN index in Feulgen (F) and PAP (P) Stain with F-P difference of 47 patients. These patients are from all sections of patients – SLT users, non users, Pre-cancer lesions, cancerous lesions**

| Sr. No | MN index Feulgen | MN index PAP | F-P | Sr. No | MN index Feulgen | MN index PAP | F-P |
|---|---|---|---|---|---|---|---|
| 1 | 2 | 1.7 | 0.3 | 31 | 3.1 | 3.2 | −0.1 |
| 2 | 5.4 | 3.2 | 2.2 | 32 | 1.1 | 1.6 | −0.5 |
| 3 | 3.4 | 2.9 | 0.5 | 33 | 1.4 | 0.9 | 0.5 |
| 4 | 3 | 1.8 | 1.2 | 34 | 4.5 | 12.8 | −8.3 |
| 5 | 1.7 | 3.3 | −1.6 | 35 | 0.8 | 1.6 | −0.8 |
| 6 | 1.9 | 1.3 | 0.6 | 36 | 2.6 | 5.3 | −2.7 |
| 7 | 2.7 | 2.2 | 0.5 | 37 | 3.5 | 4.3 | −0.8 |
| 8 | 4.9 | 2.3 | 2.6 | 38 | 1.3 | 4.5 | −3.2 |
| 9 | 3.7 | 1.9 | 1.8 | 39 | 0.8 | 1.8 | −1 |
| 10 | 1.6 | 1.1 | 0.5 | 40 | 1.4 | 0.9 | 0.5 |
| 11 | 2.4 | 2.5 | −0.1 | 41 | 2.3 | 2.1 | 0.2 |
| 12 | 2 | 2.7 | −0.7 | 42 | 12.87 | 17.36 | −4.49 |
| 13 | 1.4 | 2.5 | −1.1 | 43 | 10.19 | 14.15 | −3.96 |
| 14 | 2.1 | 1.5 | 0.6 | 44 | 14.54 | 21 | −6.46 |
| 15 | 2.9 | 1.9 | 1 | 45 | 8.25 | 10.9 | −2.65 |
| 16 | 3.1 | 3.1 | 0 | 46 | 8.65 | 9.34 | −0.69 |
| 17 | 1.8 | 1.7 | 0.1 | 47 | 11.8 | 10.61 | 1.19 |
| 18 | 3.4 | 3 | 0.4 | | | | |
| 19 | 4 | 2.3 | 1.7 | Total | 171.8 | 193.46 | |
| 20 | 2.3 | 2.5 | −0.2 | Average | 3.655319 | 4.11617 | |
| 21 | 0.8 | 1.2 | −0.4 | Std Dev | 3.286454 | 4.479435 | |
| 22 | 4.3 | 6.2 | −1.9 | | | | |
| 23 | 2.1 | 3.2 | −1.1 | | | | |
| 24 | 2 | 2.3 | −0.3 | | | | |
| 25 | 1.9 | 1 | 0.9 | | | | |
| 26 | 1 | 1 | 0 | | | | |
| 27 | 1.9 | 1.8 | 0.1 | | | | |
| 28 | 8.7 | 4.6 | 4.1 | | | | |
| 29 | 2.8 | 2.6 | 0.2 | | | | |
| 30 | 1.5 | 1.8 | −0.3 | | | | |

to tobacco occur in a basal layer which has dividing cells. MN formation due to lack of integration in a single nucleus occur here, and subsequently, these cells migrate upward. During migration, some cells die and fail to reach up. One counts MN in these superficial shedded cells.

There are several techniques to stain MN.[11,13] These are either DNA specific (Feulgen, Florescent Acridine orange, 4,6- diamini-2-phenylindole, Propidium iodide, FISH - Centromere, Tubulin) or nonspecific such as Geimsa, H and E, May Grunewald, and PAP. There are comparative studies between the two types of stain. Results vary. It cannot be overemphasized that collection and procedures in processing very much matter. We present our own method and standardization. It is unique as it uses liquid cytology and image capturing and subsequent analysis. Feulgen stain is considered to be gold standard[9,10] both these studies were for quantization of DNA.

## MN criteria

Liquid collection is unique in this study. Figures 2 and 3 show well spread epithelial cells. Traditional direct collection and spread previously caused overlaps which reduced numbers of MN cells that could be counted.

There are many chromatin bodies in various stages in the cytoplasm. They are variously named as broken eggs, pyknosis, keryorrhexis, and karyolitic. These are not MN.

The combined criteria of Heddle and Countryman (1976) and Tolbert's *et al*.[5] are what we have considered. Criteria are simple - the nuclear material which is rounded or oval, smaller than nucleus (<1/3 of nucleus). The intensity of stain is not more than main nucleus, and the nuclear "body" is not touching nucleus. MN is plainly in the cytoplasm. The "nuclear" bodies touching cell boundary has to be carefully evaluated as it could be artefact, as demonstrated in Figure 3. While counting MN the principle is that, when in doubt do not count that material as MN. This way one avoids counting false positive MN.

Tobacco chewing is widespread in India and South East Asia. For any technique that one wants to propagate on wider scale, it has to be low capital intensive, reproducible at the hands of average skilled technicians, technologically reliable, affordable, and useful. Table 1 shows that statistically PAP stain and Feulgen for the purpose of identifying are comparable. PAP stain, which is widely used for cervical smear screening, is widely available and can be used for mass screening purpose.

Buccal smear (MN) as done in this study meets the criteria. It can be adopted in regular pathology laboratory with an investment of about $ 100-200 should be adequate (Rs. 70–120,000) with the use of image analyzer with software.

## Value and limitation of MN-based screening

MN has been used to study the cytotoxic and genotoxic effect of various substances; this is as a research tool at present. Optimal use of MN index is not for diagnostic of early cancer but for indicative of the trend during screening. Like all screening, the results are pointers and have to be used judiciously. So far buccal MN screening, as epidemiology, sociology, and clinical diagnostic laboratory tool have not been widely adopted. PAP (popularly known as PAP stain) has been widely accepted and used for cervical cancer screening. For oral - buccal mucosa - there has not been any such structured program. This is particularly so in oral lesions related to tobacco (smoking, chewing, and even in snuff users).

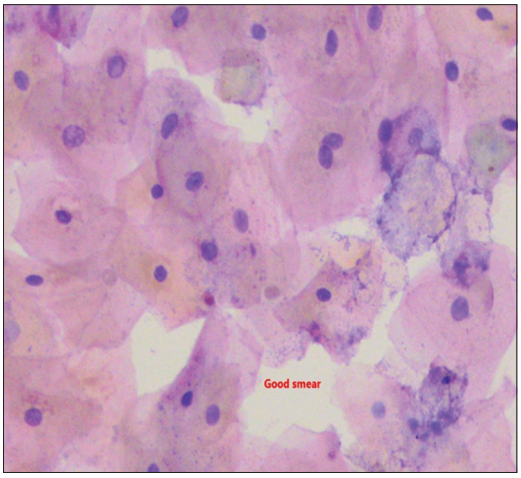


**Figure 2:** Representative Papanicolaou staining of buccal epithelial cells

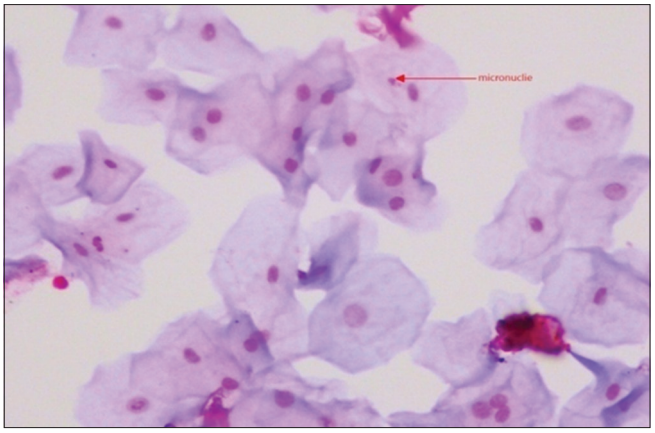


**Figure 3:** Feulgen staining of an evenly spread good smear

The studies, however, vary in many details, consistency. Hardly any paper worth its merit shows sample size of over 100. One needs to establish a range for various groups and geographic area. In most studies, this is insufficient for using as comparative guidelines in clinical practice.

In this paper, we have defined MN Index as per 100 cells. However, for communication to patients, one can use MN per 1000 cells. This will give meaningful numbers.

### Potential of MN Study

The MN study, so far, has not been used to its full potential

1. MN could be useful indicator to forewarn users[14] of possible danger trend and hopefully persuade the user to quit SLT habit. For this, one has to establish a local cut off limits.
2. Educational and social camps can and should be reinforced with MN reporting.
3. MN studies could be of potential objective help in toxicity studies and drug development.

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