A comparative study for qualitative and quantitative analysis of light and fluorescence microscopy stains in oral premalignant and malignant lesions

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ABSTRACT

Objectives: The present study highlights the use and benefit of fluorescence microscopy stains in easy diagnosis of oral premalignant and malignant lesions. This study was carried out to evaluate the efficacy of light microscopy using Papanicolaou stain (PAP) and May Grunwald Giemsa (MGG) stains and fluorescence microscopy using acridine orange (AO) and 4’,6-diamidino-2-phenyl indole (DAPI) stains. All smears have been qualitatively and quantitatively analyzed in premalignant and malignant lesions using cytology and cytomorphometry.

Materials and Methods: The subjects included in this study were selected from patients suffering from premalignant and clinically suspicious malignant lesions. Specimens were collected from the most representative site in study groups and normal buccal mucosa in the control group. The smears were evaluated under fluorescence microscopy for AO and DAPI and under light microscopy for conventional PAP and MGG. We studied 5 HPF to compare the number of normal cells to dysplastic cells. These were then subjected for cytomorphometric and statistical analysis.

Results: Assessment of quality using quality index revealed that PAP has better diagnostic efficiency as compared to MGG because the quality index of PAP was 0.87 and that of MGG was 0.74, the quality index of AO was 0.9, and that of DAPI was found to be 0.82, hence proving AO to be better in comparison with DAPI.

Conclusion: We found that among all the four stains used AO is reliable, has greater efficacy, and can be easily performed with less cost. Despite the difficulties mentioned in this study, the progress in the field of fluorescence microscopy is expected to be rapid in the following years.

Keywords: 4’,6-diamidino-2-phenyl indole, Acridine orange, Fluorescence microscopy, Premalignant conditions, Premalignant lesions

INTRODUCTION

Since the late 17th century, microscopes have yielded innumerable glimpses of a minute world that cannot be seen by the naked eye.[1] Today, the instrument is often used as the definitive symbol of scientific investigation.[2] Microscopes have continued to improve since they were first invented and used by early scientists like Anthony Leeuwenhoek to observe bacteria, yeast, and blood cells. Each type of microscope offers unique advantages and disadvantages, and all have become essential tools to the progress of science.[3]
The optical microscope, often referred to as the “light microscope,” is a type of microscope which uses visible light and a system of lenses to magnify images of small samples. But living biological material suffers from a lack of contrast, for there is little variation in light absorption by the cellular components that we wish to distinguish. The emphasis with more advanced microscopic techniques is to introduce contrast optically, as in dark field, phase contrast, and differential interference contrast microscopy. Fluorescent microscopy has the ability to isolate specific regions of the specimen using fluorescent dye that has made it an essential tool for some laboratory situations.[3]

Fluorescence microscopy has become an essential tool in biology as well as in material science as it has attributes that are not readily available in other optical microscopy techniques. The use of an array of fluorochromes has made it possible to identify cells and submicroscopic cellular components and entities with a high degree of specificity amid nonfluorescing material.[3]

For routine diagnostic cytology using light microscopy Papanicolaou stain (PAP) is recommended, as it stains nuclear chromatin well, gives good differential cytoplasmic counterstaining, and produces good cytoplasmic transparency. May Grunwald Giemsa (MGG) stain was first used for peripheral blood smears, but there have been many studies using MGG stain in oral cytology.[4]

For fluorescence microscopy, stains generally used include acridine orange (AO) and 4’,6-diamidino-2-phenyl indole (DAPI). The AO fluorescence method has been elaborated for the entire field of exfoliative cytology as it binds to the nuclear DNA and cytoplasmic RNA. This is largely due to the work of Bertalanffy. AO stain shows a wide variation depending on factors such as concentration of the dye, pH, ionic balance, duration of staining, and fixation as well as on properties of the tissues under investigation.[5]

DAPI forms a fluorescent complex by attaching in the minor groove of A-T rich sequences of DNA. It also forms non fluorescent intercalative complexes with double-stranded nucleic acids. The physicochemical properties of the dye and its complexes with nucleic acids and history of the development of this dye can be evaluated as a biological stain.[6]

Aims and objective

This study was carried out to evaluate the efficiency of light microscopy using PAP and MGG stains and fluorescence microscopy using AO and DAPI stains. All smears have been qualitatively and quantitatively analyzed in premalignant and malignant lesions using cytology and cytomorphometry.

MATERIAL AND METHODS

The present study involved:

a. Sample selection
b. Qualitative analysis of stains using light microscopy and fluorescence microscopy in cytosmears
c. Cytomorphometric analysis using light microscopy and fluorescence microscopy.

Sample size of the present study comprised of 80 patients divided into the following groups:

- Group 1 – 20 patients with no lesions and without habits (Control Group)
- Group 2 – 20 patients with tobacco chewing and/or smoking habits but no lesions
- Group 3 – 20 patients with tobacco chewing and/or smoking habits and lesions. (Tobacco pouch keratosis and homogenous leukoplakia)
- Group 4 – 20 patients with tobacco chewing and/or smoking habit with suspected oral cancer (Speckled leukoplakia, erythroplakia, and ulcerated lesions which were confirmed histopathologically as ca in situ/scc).

The subjects included in this study were selected from the patients visiting YMT dental college and suffering from premalignant and clinically suspicious malignant lesions. Specimens were collected using a candy stick from the most representative site in study groups and normal buccal mucosa in the control group. The smears were evaluated under fluorescence microscopy for AO and DAPI and under light microscopy for conventional. These were then subjected for cytomorphometric analysis. Statistical analysis was carried Papanicolaou and MGG out for the same.

Inclusion criteria

1. The patients comprising the study groups were above 18 years of age
2. The patients in the control group did not have any clinically observable lesions and habits.

Exclusion criteria

The following criteria were excluded from the study:

1. Patients with any other mucosal lesions
2. Patients with systemic illness.

Slides were examined under ×10 and ×40 magnification using light microscope for PAP and MGG stained slides and fluorescence microscope for AO and DAPI stained slides. For all the slides, scoring was done at ×40 in five high power fields and a mean cytoplasmic area (CA), nuclear area (NA), and ratio of NA and CA were calculated and evaluated by statistical analysis. Cytomorphometric analysis was done
using SPSS software. The quality of the stains used was also assessed using quality index.

### Assessment criteria for quality index (Idris and Hussain, 2009)

| Score | 1 | 2 | 3 |
|-------|---|---|---|
| Slide quality | Background | Hemorrhage/debris | Clean |
| Cell morphology (cytoplasmic details) | Not preserved | Diffuse | Well preserved |
| Overall staining | Bad | Moderate | Good |
| Nuclear characteristics (boundaries) | Smudgy | Crisp |

Maximum score for a single case = 10

Maximum possible score = 10 × Sample size

= 10 × 80

= 800.

Quality index = Score obtained/maximun possible score.

## RESULTS

- Since $P$-value for all the three parameters is $>0.05$, it indicates no significant difference between PAP and MGG in Group 1.

| Group 2 | Stain | $n$ | Mean and Std Deviation | $P$-value |
|---------|-------|-----|------------------------|-----------|
| NA      | PAP   | 20  | 55.650±14.90417        | 0.000 *   |
|         | MGG   | 20  | 80.200±20.15362        | 0.000*    |
| CA      | PAP   | 20  | 1567.500±585.90582     | 0.915     |
|         | MGG   | 20  | 1587.550±591.46433     | 0.915     |
| Ratio   | PAP   | 20  | 0.0494±0.04648         | 0.441     |
|         | MGG   | 20  | 0.0591±0.03033         | 0.441     |

NA: Nuclear area, CA: Cytoplasmic area, PAP: Papanicolaou stain, MGG: May Grunwald giemsa

- $P$-value for the NA, CA is $>0.05$ indicating no significant difference between PAP and MGG NA and CA for in group 4.
- $P$-value for the N: C (Nuclear: cytoplasmic) ratio is $<0.05$ indicating significant difference between PAP and MGG for ratio in Group 4.
- $P$-value for all the parameters is $>0.05$, this indicates no significant difference between stains of light microscopy against fluorescence microscopy.

## DISCUSSION

Microscopes play a pivotal role in many aspects of our society. Over the past few decades, different microscopes with better properties have evolved. The main aim of this study was to explore the efficacy of the fluorescence microscope and study newer stains in comparison to the gold standard, the conventional microscope and its stains in premalignant and malignant lesions using cytology as the screening modality.

In the present study, cytosmears were obtained from 80 patients, of which 58 were males and 22 were females. The age of these patients ranged from 18 to 72 years, of which 82% were above the age of 45 years. Patients were grouped into categories depending on their habits and lesions. Graph II shows distribution of the various lesions in Group III and Group IV.

In our study, fluorescence and light microscopes were compared for the assessment of stains using exfoliative cytology. In fluorescence microscopy, as the specimens are self-illuminated by internal light it shows bright objects in vivid color against a dark background.

When all the four stains were compared, the quality index of fluorescence stains was greater than light microscopy stains including contrast, clarity, and resolution. The time consumed for staining the smear is less than PAP and MGG. One important disadvantage of fluorescence stains is that the time for which the slides can be evaluated is only 2–3 h whereas slides stained with PAP and MGG retain the stain longer [Table 1]. Furthermore, the stains used and the fluorescence microscope are more expensive.

Our study found that the fluorescence technique is faster and simpler (requiring only 10 s for the preparation of
smears which was a technique followed by Bertlantfly, and expedites the process. Study conducted by Marks and Goodwin (1962) states the same, though their procedure using AO takes 7 min for preparation of smears.

The present study found PAP to be more practical than MGG as it is easy to process and transport. Qualitative analysis revealed that PAP is easier to read than MGG as it gives differentiation of cell layers based on color. It also has a better resolution, gives well defined cytoplasmic boundaries and is less time consuming as compared to MGG [Table 1]. For PAP, fixation is done with 95% ethyl alcohol which is bactericidal and maintains morphological integrity of the cells. PAP consists of hematoxylin as the nuclear stain which stains the nuclear DNA. It also consists of two counterstains due to which the cytoplasm appears transparent and the cell boundaries are well demarcated. On the contrary, we found MGG difficult to read as cellular debris masks cellular details.

The slides have to be air dried and then fixed with methanol which is a tedious process. Our study was consistent with Roberts (1997) and Guzman et al. (2003). Assessment of quality using quality index revealed that PAP has better diagnostic efficiency as compared to MGG because the quality index of PAP was 0.87 and that of MGG was 0.74 considering multiple parameters [Table 2]. This view has been supported by studies done by Idris and Hussain in (2009) and Belgaumi et al. in (2013).

Between the two fluorescent dyes, the quality index of AO was 0.9 and that of DAPI was found to be 0.82, hence proving AO to be better in comparison with DAPI [Table 2]. Furthermore, AO displays sharp and clear nuclear and cytoplasmic boundaries, whereas when DAPI is visualized with the blue green filter it gives cytoplasm hazy boundaries. Furthermore, DAPI is more time consuming and expensive in comparison to AO.

Table 1: Qualitative analysis of stains.

| Features                                      | PAP                  | MGG                  | DAPI                  | AO                  |
|-----------------------------------------------|----------------------|----------------------|----------------------|---------------------|
| Differentiation of epithelial layers based on color | Stains with different colors for different layers | Cannot be distinguished based on color difference | Cannot be distinguished based on color difference | Cannot be distinguished based on color difference |
| Superficial layer – orange                   |                      |                      |                      |                     |
| Intermediate layer – pink                    |                      |                      |                      |                     |
| Basal layer – blue                           | Can be distinguished | Can be distinguished | Questionable         | Can be distinguished |
| Differentiation of layers of epithelium based on morphology | Can be distinguished | Can be distinguished | Questionable         | Can be distinguished |
| Contrasting                                   | Less than DAPI and AO| Less than DAPI and AO| Better               | Better than PAP, MGG and DAPI |
| Clarity                                       | Less than DAPI and AO| Hazy boundaries      | Better than MGG but less than PAP and AO | Better than PAP, MGG, and DAPI |
| Resolution                                    | Good                 | Hazy                 | Hazy for cytoplasm   |                     |
| Micronuclei detection                         | Less as compared to DAPI and AO | Less as compared to DAPI and AO | Good as it is a nuclear stain and binds mainly to DNA | Good |
| Procedure                                     | Tedium               | Tedium               | Easy                 | Easy |
| Time required for staining                    | 10–15 min            | 15–20 min            | 6 min                | 10 s |
| Nuclear details                               | Good                 | Good                 | Very good            | Good |
| Cytoplasmal boundaries                        | Well defined         | Not very clear       | Hazy with blue green filter 2–3 h | Well defined 2–3 h |
| Time for which stain is retained              | Longer than DAPI and AO | Longer than DAPI and AO | Longer than DAPI and AO |                 |
| Microscope                                    | Light                | Light                | Fluorescence         | Fluorescence |
| Expenses of the stain                         | Not very expensive   | Not very expensive   | Expensive            | Expensive |
| Expenses of the microscopic                   | Less as compared to fluorescence microscope | Less as compared to fluorescence microscope | Expensive | Expensive |

PAP: Papanicolaou stain, MGG: May grunwald giemsa, AO: Acridine orange, DAPI: 4′,6-diamidino-2-phenyl indole
We carried out cytomorphometric analysis using exfoliative cytology and have concluded from our results that, as obtaining a smear and spreading it correctly onto the slide is technique sensitive the amount of dysplastic cells accounts to a lesser number than the normal cells obtained. Also in the 5 HPFs that we studied that the number of normal cells was found to be more than dysplastic cells. This camouflages and compensates for the values of dysplastic cells as mean value for cells in 5 HPFs are considered.

Cytomorphometric analysis was done by calculating the nuclear and CA using SPSS software. Studies have also considered nuclear diameter and cytoplasmic diameter, but we feel that as the cells are polygonal and have varying shapes measuring their diameter would be inappropriate and considering NA and CA was more accurate.

AO and DAPI were used to stain smears for fluorescence microscopy. The efficacy of AO was higher than DAPI. Moreover, it was higher than light microscopy stains – PAP and MGG [Table 2]. Comparison of PAP and MGG revealed a $P > 0.05$, which indicated that it was not statistically significant for all groups [Table 3].

Comparison of DAPI and AO revealed a $P > 0.05$, which indicated that it was not statistically significant for all groups. Furthermore, cytomorphometric analysis for comparison between light and fluorescence microscopy stains did not yield statistically significant values [Table 4].

When PAP, MGG, DAPI, and AO stains were analyzed, we found that exfoliative cytology using AO is reliable and is easily performed with less cost. AO technique has been developed primarily for cytochemical demonstration of RNA and DNA. The proliferating malignant and premalignant cells which are especially rich in these are demonstrated better with AO technique. Micronuclei demonstration is the best in AO amongst all stains used [Table 1]. This can be used as an early diagnostic tool for diagnosis of preneoplastic and neoplastic lesions due to the early occurrence of micronuclei, in the affected cells, even before clinical or histopathological changes are evident. Hence, it can be used as an indicator of risk for malignant transformation. When stained with AO the cells also reveal much morphological detail, sufficient for final diagnosis. We feel that the fluorescent AO method can be used reliably for the screening of mucosal lesions and this view is also shared by Prakash et al. (2011). It can be used for screening suspicious oral lesions when biopsy is not advisable which was consistent with the study of Joshi and Kaijkar (2013).

Considering the qualitative analysis, the advantage of PAP stain in cytosmears is that it provides color differentiation

![Figure 1: Papanicolaou stain staining showing color differentiation of different layers.](image)

| Parameters           | PAP   | MGG   | AO    | DAPI  |
|----------------------|-------|-------|-------|-------|
| Background score     | 153   | 151   | 154   | 150   |
| Staining score       | 186   | 153   | 185   | 190   |
| Nuclear score        | 148   | 138   | 155   | 157   |
| Cell score           | 214   | 153   | 226   | 160   |
| Total score          | 701   | 595   | 720   | 657   |
| Quality index        | 0.87  | 0.74  | 0.9   | 0.82  |

PAP: Papanicolaou stain, MGG: May grunwald giemsa, AO: Acridine orange, DAPI: 4′,6-diamidino-2-phenyl indole

| Group 1 | Stain | $n$ | Mean and Std. Deviation | $P$-value |
|---------|-------|-----|-------------------------|-----------|
| NA      | PAP   | 20  | 55.9620±4.86970         | 0.697     |
|         | MGG   | 20  | 56.5620±4.79783         | 0.697     |
| CA      | PAP   | 20  | 2272.4570±166.68102     | 0.997     |
|         | MGG   | 20  | 2272.6710±166.64428     | 0.997     |
| Ratio   | PAP   | 20  | 0.0246±0.00125          | 0.537     |
|         | MGG   | 20  | 0.0249±0.00134          | 0.537     |

*Statistically significant $P$-value. NA: Nuclear area, CA: Cytoplasmic area, PAP: Papanicolaou stain, MGG: May grunwald giemsa

| $n$ | Mean and Std. Deviation | $P$-value |
|-----|-------------------------|-----------|
| NA  | PAP and MGG             | 160       | 65.8420±22.43041        | 0.701     |
|     | DAPI and AO             | 160       | 66.7182±18.18267        | 0.701     |
| CA  | PAP and MGG             | 160       | 2165.5441±703.52054     | 0.345     |
|     | DAPI and AO             | 160       | 2236.9951±645.92123     | 0.345     |
| Ratio| PAP and MGG             | 160       | 0.0350±0.02455          | 0.206     |
|     | DAPI and AO             | 160       | 0.0321±0.01437          | 0.206     |

PAP: Papanicolaou stain, MGG: May grunwald giemsa, AO: Acridine orange, DAPI: 4′,6-diamidino-2-phenyl indole, NA: Nuclear area, CA: Cytoplasmic area

Table 4: Cytomorphometric analysis of parameters for light and fluorescence microscopy using ANOVA and Tukey’s test.
of layers of epithelial cells in comparison with MGG, AO and DAPI [Figure 1]. MGG stain displays hazy nuclear and cytoplasmic details making its interpretation difficult in comparison with PAP and AO [Figure 2]. AO stained cytosmear on the contrary displays clear nuclear and cytoplasmic details [Figure 3]. DAPI is a useful nuclear stain and provides hazy cytoplasmic boundaries [Figure 4].

This study highlights the efficiency of the fluorescence microscopy in comparison with the optical microscopy using various stains. Fluorescence microscopy when applied in cytology is a simple and non-invasive technique. It is also very useful in those cases where biopsy is contraindicated. However, with a few limitations like evaluation by a single examiner, subjectivity in scoring, the sensitivity and specificity of the various stains used and staining technique, it needs to be further evaluated. Within the limitations of the study, the AO staining technique was found to give better results amongst all stains. Keeping in mind the added advantages of better morphological details and speed of the technique, the study supports the idea of utilizing this method for early detection of oral lesions, especially in mass screening programs. Large scale studies can be carried out to further confirm the efficacy of this procedure.

**CONCLUSION**

We found that amongst all the four stains used AO is reliable, has greater efficacy and can be easily performed with less cost. However, further studies with a greater sample size should be done to establish concrete evidence. Despite the difficulties mentioned in this study, the progress in the field of fluorescence microscopy is expected to be rapid in the following years. New research evidence is continuously being generated, it seems that the journey is long and this is just the beginning.

**COMPETING INTERESTS STATEMENT BY ALL AUTHORS**

No competing interests.

**AUTHORSHIP STATEMENT BY ALL AUTHORS**

Both authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript.

**ETHICS STATEMENT BY ALL AUTHORS**

1) This material is the authors’ own original work, which has not been previously published elsewhere. 2) The paper is not currently being considered for publication elsewhere. 3) The paper reflects the authors’ own research and analysis in a truthful and complete manner.

**LIST OF ABBREVIATIONS (In Alphabetic Order)**

AO – Acridine orange
DAPI – 4,6 di amidino 2 phenyl indole
MGG – May Grunwald Giemsa
PAP – Papanicoula stain.

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*Figure 2: May grunwald giemsa displaying hazy nuclear and cytoplasmic details.*

*Figure 3: Acridine orange stain – displaying increase in size of nuclei.*

*Figure 4: 4’,6-diamidino-2-phenyl indole: The nuclear stain with hazy cytoplasmic boundaries.*
EDITORIAL/PEER-REVIEW STATEMENT

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