

## ORIGINAL ARTICLE

# Confocal microscopy and exfoliative cytology

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## ABSTRACT

**Context:** Early detection of potentially malignant lesions and invasive squamous-cell carcinoma in the oral cavity could be greatly improved through techniques that permit visualization of subtle cellular changes indicative of the neoplastic transformation process. One such technique is confocal microscopy. Combining rapidity with reliability, an innovative idea has been put forward using confocal microscope in exfoliative cytology. **Aims:** The main objective of this study was to assess confocal microscopy for cytological diagnosis and the results were compared with that of the standard PAP stain. **Settings and Design:** Confocal microscope, acridine orange (AO) stain, PAP (Papanicolaou) stain. The study was designed to assess confocal microscopy for cytological diagnosis. In the process, smears of patients with (clinically diagnosed and/or suspected) oral squamous cell carcinoma as well as those of controls (normal people) were stained with acridine orange and observed under confocal microscope. The results were compared with those of the standard PAP method. **Materials and Methods:** Samples of buccal mucosa smears from normal patients and squamous cell carcinoma patients were made, fixed in 100% alcohol, followed by AO staining. The corresponding set of smears was stained with PAP stain using rapid PAP stain kit. The results obtained were compared with those obtained with AO confocal microscopy. **Results:** The study had shown nuclear changes (malignant cells) in the smears of squamous cell carcinoma patients as increased intensity of fluorescence of the nucleus, when observed under confocal microscope. Acridine orange confocal microscopy showed good amount of sensitivity and specificity (93%) in identifying malignant cells in exfoliative cytological smears. **Conclusion:** Confocal microscopy was found to have good sensitivity in the identification of cancer (malignant) cells in exfoliative cytology, at par with the PAP method. The rapidity of processing and screening a specimen resulted in saving of time. It added a certain amount of objectivity to the process of arriving at a diagnosis.

**Key words:** Acridine orange staining, squamous cell carcinoma, confocal microscopy, exfoliative cytology, PAP (Papanicolaou) stain

## INTRODUCTION

Early detection of potentially malignant lesions and invasive squamous-cell carcinoma in the oral cavity could be greatly improved through techniques that permit visualization of subtle cellular changes indicative of the neoplastic transformation process. One such technique is confocal microscopy which

offers several advantages over conventional optical microscopy. Combining rapidity with reliability, an innovative idea has been put forward using confocal microscope in exfoliative cytology. The main aim and objective of the present study was to assess confocal microscopy for cytological diagnosis and the results were compared with that of the standard PAP stain.

## MATERIALS AND METHODS

Samples of buccal mucosa smears from 15 normal patients and 15 oral squamous cell carcinoma (clinically diagnosed and/or suspected) patients were made by scraping with flat wooden sticks (exfoliative cytology). The smears were fixed in 100% alcohol for 15 minutes, followed by acridine orange (AO) staining as described by Von Bertalanffy *et al.* The technique

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for AO staining followed was as follows: The fixed smears were passed through descending grades of alcohol; then rinsed for few seconds in 1% acetic acid and washed in two changes of distilled water for about 1 minute; followed by staining in 0.01% AO for 3 minutes, de-stained in the phosphate buffer solution for 1 minute; differentiated in 0.1M Calcium Chloride solution for 30 seconds to 1 minute (The nuclei should be clearly outlined); excess Calcium Chloride was removed by washing with phosphate buffer solution and mounted with cover slip in a drop of phosphate buffer solution.<sup>[1-3]</sup> Smears stained with AO were examined under confocal microscope.

The apparatus used in our study was Zeiss 510 LSM module Laser Scanning Confocal Microscope. Smears stained with AO were screened using 10× and 20× objectives. Morphological detail was studied with 40× objective and the microphotographs were taken. The absorption range was 488 nm, blue, and the emission range was 505 nm, green. Different types of cells – normal, atypical, and malignant were studied.

The corresponding set of smears were stained with PAP stain using rapid PAP stain kit.<sup>[4,5]</sup> The results obtained were compared with those obtained with AO confocal microscopy.

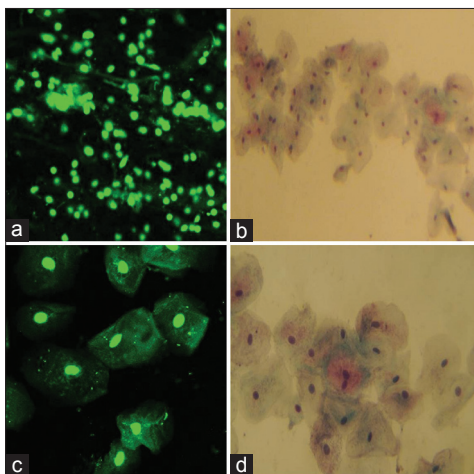
## RESULTS

Our study had shown nuclear changes in the smears of squamous cell carcinoma patients (cases); stained with AO; under confocal microscope. Literature revealed that nuclear changes were the most significant morphologic criteria for malignancy. Similarly in our study, in concurrent to literature, the consistent change seen in atypia – increase in the nuclear material – was observed in the smears of carcinoma cases as

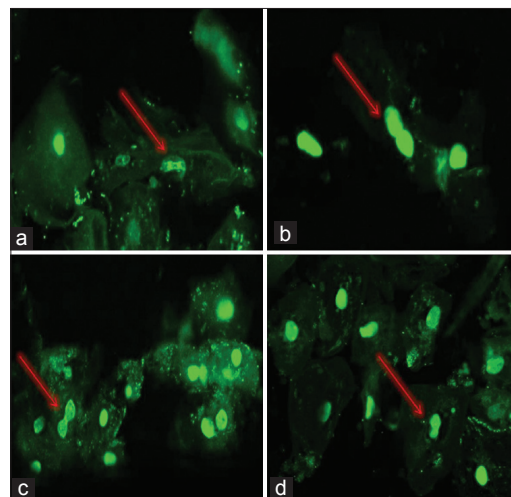
increased intensity of fluorescence of the nucleus, regardless of color staining characteristics. The cytoplasm of atypical cells was dull olive green and the nuclei had a green brilliance which produced an aura or glow that emanated from or above the nucleus [Figure 1]. Numerous mitotic figures, at different stages of mitosis, were also seen. Irregular nuclei were seen in malignant cells [Figure 2]. Although the cytoplasm of the cells in the smears of normal patients (controls) appeared green and the nuclei appeared bright, the brilliance was smooth and remained within the nucleus when observed under confocal microscope [Figure 3].

The results obtained with fluorescence microscopy were compared with the parallel series of papanicolaou smears of both normal (controls) and squamous cell carcinoma (cases) patients. Fourteen carcinoma cases (93%) were correctly diagnosed by AO confocal microscopy whereas thirteen carcinoma cases (87%) were correctly diagnosed by PAP method. One case (7%) was falsely diagnosed as negative by both AO confocal microscopy and PAP methods whereas one case that was falsely diagnosed as negative by PAP method was correctly diagnosed by AO confocal microscopy. Fourteen smears (93%) of normal patients were correctly diagnosed by both the methods where as one case (7%) was falsely diagnosed as positive by both AO confocal microscopy and PAP methods [Table 1]. It may be assumed that the exfoliated cells seen were hyperplastic and therefore fluoresced brightly when observed under confocal microscope.

The results showed that the validity of exfoliative cytology under AO confocal microscopy is at par (as good as) with that of the PAP stained smears seen under compound microscope [Table 2].



**Figure 1:** Patients with clinically diagnosed and/or suspected squamous cell carcinoma -.cases. (a,c) Acridine orange confocal microscopy, ×200 and ×400, respectively, showing the cells with increased intensity of fluorescence of the nucleus with green brilliance which produced an aura or glow that emanated from or above the nucleus, suggestive of malignant cells. (b,d) PAP staining under compound microscope, ×100 and ×400, respectively, showing abnormal mitotic figures



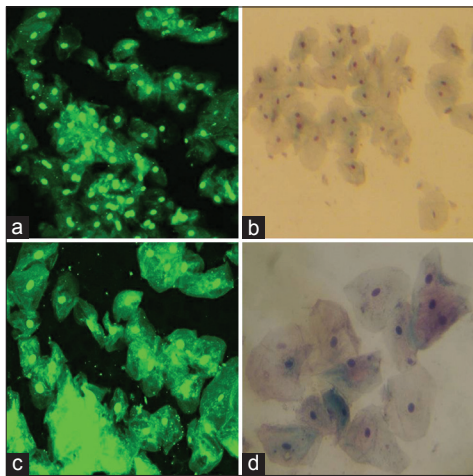
**Figure 2:** Patients with clinically diagnosed and/or suspected squamous cell carcinoma -.cases. (a-d) Acridine orange confocal microscopy, ×400, showing numerous abnormal mitotic figures at different stages of mitosis. a shows anaphase, b shows starting of telophase, c shows middle of telophase, and d shows ending of telophase of mitosis compound microscope, ×100 and ×400, respectively, showing abnormal mitotic figures

**DISCUSSION**

Exfoliative cytology is a diagnostic procedure which has been generally accepted, is growing rapidly in importance as a means of early diagnosis of cancer.<sup>[6]</sup> The diagnosis of malignant cell using cytological smears, however, is in the final analysis, subjective and it is experience and familiarity with a technique that is important, not the stain used.

Von Bertalanffy *et al.*, had reviewed the literature on the role of nucleic acids in malignant cells and the theoretical considerations for the use of basic fluorochrome stains and they have suggested the use of AO in routine exfoliative

cytology. AO is a histochemical fluorochrome with a selective affinity for nucleic acids. At a concentration of 0.01% and a pH of 6, recommended by Von Bertalanffy, the DNA fluoresces yellow to whitish green and the RNA red. The differential fluorescence is due to varying degrees of polymerization of the nucleic acids and makes possible the assessment of relative amounts of RNA and DNA in the cell. The increased RNA of the malignant cell is reflected in an increased brilliance, so that the differential fluorescence of the malignant and normal cells allows a comparison of the total concentration of nucleic acids in the various cells in the preparation. In addition, the morphological features of the cell are clearly visualized.<sup>[1-3]</sup>



**Figure 3:** Normal Patients -controls. (a,c) Acridine orange confocal microscopy, x200 and x400, respectively, the cytoplasm of the cells appeared green and the nuclei appeared bright but the brilliance was smooth and remained within the nucleus, suggestive of normal cells. (b,d) PAP staining under compound microscope, x100 and x400, respectively

The PAP technique, developed originally to investigate gynecological cancer, has been extensively applied to material from various sources and has collected a voluminous literature testifying to its experience. The papanicolaou technique was introduced by Papanicolaou in 1942. Papanicolaou stain which has become the most popular stain for gynecological cytology was originally developed to demonstrate the cyclical changes that take place in the squamous epithelium of the female genital tract in response to alteration in hormone levels. Harris hematoxylin is the optimum nuclear stain and the combination of OG 6 and EA 50 give the subtle range of green, blue, and pink hues to the cell cytoplasm. PAP stain provides a good differential stain and as a result is widely used for other routine cytology smears. The cytoplasm of the superficial cells appear pink, cytoplasm of intermediate cells appears pale greenish-blue, cytoplasm of parabasal cells appear deep greenish-blue, and nuclei appear dark blue.<sup>[4,5]</sup>

**Table 1: Results of AO confocal microscopy and PAP for cases (squamous cell carcinoma) and controls (normal)**

	Cases		Controls	
	AO confocal microscopy	PAP	AO confocal microscopy	PAP
Total No	15	15	15	15
True Positives	14	13	0	0
True Negatives	0	0	14	14
False Positives	0	0	01	01
False Negatives	01	02	0	0
% that are correctly diagnosed	93	87	93	93

AO: Acridine orange, PAP: Papanicolaou stain

Confocal microscope, pioneered by Marvin minsky in 1955, performs a point-by-point construction by focusing a point of light sequentially across the specimen and then collecting some of the returning rays. Currently, the confocal microscope is upgraded with combination of lasers that can be coupled to the fiber optics of the scanning unit to increase the number of excitation wavelengths. Powerful software that display and analyze 3-D data are added, making it an useful tool with the capability to quickly provide information about the biochemical and morphological changes that occur as tissue becomes neoplastic. The most important feature of confocal microscope is the capability of isolating and collecting a plane of focus within a sample, thus eliminating the out-of-focus “haze” normally seen with a fluorescent sample. Fine detail is often obscured by the haze and cannot be detected in a non-confocal, fluorescent microscope. In

**Table 2: Comparison of AO confocal microscopy and PAP**

	Sensitivity %	Specificity %	Positive predictive value %	Negative predictive value %	Likelihood ratio positive	Likelihood ratio negative
AO confocal microscopy	93	93	100	100	1.01	0.99
PAP	87	93	100	100	0.95	0.92

AO: Acridine orange, PAP: Papanicolaou stain



the present study, confocal microscopy has been applied to cytology using acridine group of stains which was based on the affinity of the basic fluorochrome dyes for the nucleic acids, logical exploitation of a well-established feature of the malignant cell.<sup>[7-12]</sup>

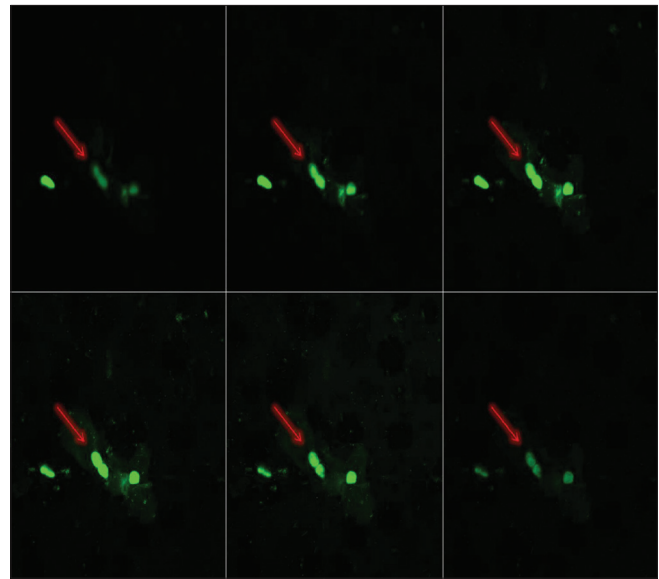
In literature, studies of the neoplastic cell had shown an increase in the total nucleic acids. This increase is characteristic of cells showing a high protein synthesis and is seen in embryonic, regenerating, secretory and malignant cells. Increase in DNA, found in the chromatin of the nucleus, is seen in cells undergoing mitosis and polyploidy characteristic of advanced cancer. Increased DNA in the nucleus of an atypical cell, which demonstrates an increased intensity of fluorescence, is the method for screening atypia without regard for morphologic comparison. Nuclear changes are the most significant morphologic criteria for malignancy. In addition, presence of irregular nuclei and mitotic figures in the smears add to the criteria for malignancy.<sup>[6,13,14]</sup>

The present study was undertaken in search of a technique which combined rapidity with reliability. AO Confocal Microscopy compared favorably with the Papanicolaou technique which was observed in the smears of (clinically diagnosed and/or suspected) squamous cell carcinoma cases as well as those of normal patients' smears. The technique was more rapid than the older methods and the present investigation confirmed these findings. The extreme brilliance of malignant cells in the smears of carcinoma patients was most striking and in the majority of the cases reported above, the correct diagnosis was made practically at the first glance. Numerous mitotic figures, at different stages of mitosis, were also seen. Irregular nuclei were seen in malignant cells.

A more important finding was the greater sensitivity of the AO confocal microscopy technique which was at par with the standard PAP method and the results have confirmed it. This was due to the clear differentiation between normal and malignant cells where the atypical cells show dull olive green cytoplasm and the nuclei have a green brilliance which produced an aura or glow that emanated from or above the nucleus. Although the cytoplasm of the normal cells appears green and the nuclei appear bright, the brilliance was smooth and remained within the nucleus when observed under confocal microscope.

The technique was more rapid than the older methods and the present investigation confirmed these findings. The staining time was 6 minutes as against the 10 minutes required by the Papanicolaou stain used in the laboratory. The average screening time was under five minutes. Moreover, the dark background of the AO preparation was less tiring to the eyes and, in addition to the screening of more specimens in a given time the pathologist can spend longer hours at the microscope without undue fatigue. Apart from the above, added advantages are high-resolution

images are obtained, has the ability to collect serial optical sections [Figure 4], and valuable tool for 3-D reconstruction [Table 3].<sup>[8,11,12]</sup> Last, but not least, the breath-taking beauty of the preparations relieves the monotony of routine work.



**Figure 4:** Optical serial sections showing the presence of same intensity of the nucleus in all the sections indicating same cell under division. Optical serial sections are helpful in distinguishing a dividing cell from an overlapping cell. Variation of intensity of the nuclei indicates overlapping of cells (Acridine orange confocal microscopy,  $\times 400$ )

**Table 3: Comparison between PAP - Compound microscopy and Acridine Orange Confocal Microscopy**

PAP-compound microscopy	Acridine orange confocal microscopy
Cytoplasm of the cells appeared transparent	Cytoplasm of the cells appeared green, making it more clearly visible
Nuclei of the cells appeared blue	Nuclei of the cells appeared green and the intensity of the fluorescence depends on the type of the cell-normal/atypia/malignant
Resolution is less when compared with Confocal Microscopy	High resolution
White background-eyes get strained easily	Black background-less tiring to the eyes
3-D reconstruction is not achieved here	3-D reconstruction is achieved here
Resolving the overlapping cells is difficult	Overlapping of the cells can be resolved by optical serial sections and 3-D images <sup>[8,11,12]</sup>
Differentiation of cells to which layer they belong to can be made due to differential cytoplasmic staining	Differentiation cannot be made
Equipment is not expensive as it involves a light compound microscope	Equipment (confocal microscope) is expensive

## CONCLUSION

Confocal microscopy was found to have good sensitivity in the identification of malignant cells in exfoliative cytology, at par (as good as) with that of the PAP method. The advantages offered by this technique are two-fold. First, the rapidity of processing and screening a specimen results in saving of time, attractive to a rapidly growing and increasingly busy department. Second, it adds a certain amount of objectivity to the process of arriving at a diagnosis. These considerations led to an attempt to assess the value of this method in routine cytological diagnosis. Immediate staining of exfoliative cytological preparations, processed by this method, can be accomplished at the dental office.

## SUMMARY

AO confocal microscopy was used to study 30 cytological specimens (15 normal and 15 squamous cell carcinoma patients) and the results were compared with the papanicolaou method. It was found to be more rapid, compared favorably with the papanicolaou technique in the examination of exfoliative cells, and was found to have good sensitivity and specificity in the identification of malignant cells in exfoliative cells.

## REFERENCES

1. Von Bertalanffy L, Masin M, Masin F. A new and rapid method for diagnosis of vaginal and cervical cancer by fluorescence microscopy. *Cancer* 1958;11:873-87.
2. Masin F, Masin M, Von Bertalanffy L. Use of acridine-orange fluorescence technique in exfoliative cytology. *Science* 1956;124:1024-5.
3. Darzynkiewicz Z. Differential staining of DNA and RNA in intact cells and isolated cell nuclei with acridine orange. *Methods Cell Biol* 1990;33:285-98.
4. Papanicolaou GN. *Atlas of Exfoliative Cytology*. Massachusetts: Harvard University Press; 1954.
5. McMillan A. The detection of genital tract infection by Papanicolaou-stained tests. *Cytopathology* 2006;17:317-22.
6. Grubb C, Crabbe JG. Fluorescence microscopy in exfoliative cytology. *Br J Cancer* 1961;15:483-8.
7. Singh A, Gopinathan KP. Confocal microscopy: A powerful technique for biological research. *Curr Sci* 1998;74:841-5.
8. Rowland RE, Nickless EM. confocal microscopy opens the door to 3-dimensional analysis of cells. *Bioscience* 2000;26:3-7.
9. Amos WB, White JG. How the confocal laser scanning microscope entered biological research. *Biology of the Cell* 2003;95:335-42.
10. Semwogerere D, Weeks ER. Confocal Microscopy. *Encyclopedia of Biomaterials and Biomedical Engineering*, London, U.K.: Taylor and Francis; 2005.
11. St Croix CM, Shand SH, Watkins SC. Confocal microscopy: Comparisons, applications, and problems. *Biotechniques* 2005;39; (Suppl 6): 2-5.
12. Wanninger A. The application of confocal microscopy and 3D imaging software in functional, evolutionary, and developmental zoology: Reconstructing myo- and neurogenesis in space and time. *Modern research and educational topics in microscopy*. Badajoz, Spain: Formatex; 2007.
13. Marks R, Goodwin AM. Comparative Evaluation of the acridine orange fluorescence and papanicolaou methods for cytodiagnosis of cancer. *Br J Cancer* 1962;16:390-9.
14. Oakland DJ. Fluorescence microscopy in colonic exfoliative cytology. *Gut* 1964;5:99-102.

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