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RESEARCH ARTICLE

Intrinsic fluorescence spectroscopy and detection of pre-invasive lesions of the cervix using portable optical device

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ABSTRACT

Objectives: To evaluate the screening potential of portable optical device based on the principle of intrinsic fluorescence spectroscopy to detect the fluorescence of FAD molecules in pre-invasive lesions of cervix and its comparison to conventional screening methods. **Methods**: A prospective cross-sectional study was conducted on 163 patients, who underwent hysterectomy for various gynecological indications. Pap smear and colposcopy was done for all the patients pre-operatively. Cervix from the hysterectomised sample was examined with the optical device, based on the principle of intrinsic fluorescence spectroscopy. The sample was then sent for histopathological examination. The findings of pap smear, colposcopy and fluorescence spectroscopy were correlated with histopathology (gold standard) to find the accuracy of these methods. **Results**: The sensitivity and specificity for optical device using co-polarized light was 90.5% and 90.9% respectively and for cross polarized light it was 81.1% and 87.3% respectively. The above mentioned results were better for optical device using polarized light as compared to conventional pap smear results. **Conclusion:** Due to the high sensitivity and specificity, the designed optical device has a potential to be a promising screening tool which can provide see-to-treat protocol and early intervention for pre invasive lesions of cervix.

Keywords: Intrinsic fluorescence spectroscopy, flavin adenine dinucleotide, pap smear, colposcopy, histopathology.

Cancer cervix is the 2nd most common cancer amongst females in India with crude incidence rate of 14.9 per 100,000. India has 469.1 million female population aged >15 years who are at risk of developing cancer cervix annually. Each year 96,922 women are diagnosed with cancer cervix and 60,078 women die of the disease[1]. Globally, cancer cervix is the 4th most common cancer in women with estimated 570,000 cases and 311,000 deaths in the year 2018 [2]. Invasive cervical cancer is preceded by a long pre-invasive phase which progresses slowly and is referred as cervical intraepithelial neoplasia (CIN) [3]. The mean time interval for progression of pre cancer to invasive cancer is approximately 10 years. Thus, unlike other cancers, cervical cancer is preventable as it can be subjected to early detection by proper screening methods and treatment accordingly [4].

Various screening methods for cancer cervix in high resource settings include exfoliative cytology by conventional and liquid based methods and human papilloma virus DNA (HPV DNA) detection test. While screening by visual inspection with acetic acid (VIA) and lugol's iodine (VILI) is done in low resource settings [5]. There are certain limitations to exfoliative cytology (pap smear) test like, low sensitivity due to inadequate collection, errors during sample preparation, possibility of improper microscopic evaluation and need for trained health professionals in low and middle income countries (LMIC) like India [6-8]. Aided visual

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inspection methods like VIA and VILI are simple, requiring minimal expertise. However its main disadvantage, apart from being inherently subjective, is the low specificity leading to over diagnosis and its inability to screen the endocervical pathologies [9].

Prompted by the requirement of an effective screening method, optical imaging has been under research in India and abroad [10-12]. Biophotonics is the fusion of biology and photonics which deals with interaction of light with biological tissues and involves a new dimension of research in screening and early diagnosis of diseases [13]. Robert Alfano did pioneering works in the field of optical spectroscopy and his innovative application of spectroscopic techniques like fluorescence spectroscopy, Raman spectroscopy, stokes shift emission spectroscopy for differentiation between normal and cancerous tissues are a major contribution in this field [14-16]. Dr. Rebecca Richards- Kortum and her group examined slices of cervical tissue to see the variation of cervical fluorescence in different groups of patients. These studies indicate that there are important changes that occur with dysplasia [17-19].

Various optical techniques such as fluorescence spectroscopy are sensitive to detect the subtle morphological and biochemical changes occurring in the dysplastic cells [10]. In biological cells there are some native fluorophores known as auto-fluorophores namely Flavin Adenine Dinucleotide (FAD), Nicotinamide Adenine Dinucleotide (NADH), tryptophan and porphyrins. Furthermore, there are some extracellular structural auto-flurophores such as collagen. As the dysplastic changes occur in the cell, the concentration of these fluorophores changes. At selected wavelengths (405nm), FAD is the dominant flourophore and its fluorescence peak does not overlap with other autofluorophores. Also contribution of NADH is very negligible at this wavelength. As the disease progresses, FAD converts to its reduced form, which is non fluorescent [17-20]. These alterations result in change in fluorescent properties which is detected through emission spectrum [19, 20]. The optical device based on this principle was designed and fabricated by the research team of Indian Institute of Technology (IIT) Kanpur. Initially the device was developed as a table top arrangement and experiment was conducted on the biopsied specimens of cervix. Gradually, further modifications were made and a portable optical device was developed for community use. The present study was conducted to evaluate the screening potential of this portable optical device to detect the fluorescence of FAD molecules and its comparison to conventional screening methods using histopathology as the gold standard.

Materials and methods

A prospective cross sectional study was conducted in the Department of Obstetrics and Gynecology, Ganesh Shankar Vidyarthi Memorial Medical College (GSVM) Kanpur, in collaboration with IIT Kanpur, from February 2015 to May 2016. The study was approved by the Institutional Ethics Committee.

A total of 163 patients were included in the study. All the patients who underwent hysterectomy for various gynecological indications such as premalignant lesions, abnormal uterine bleeding or utero-cervical descent were included in the study. Those patients who underwent emergency hysterectomy, caesarean or subtotal hysterectomy were excluded from the study. Written informed consent was taken from each study participant.

Pap smear (exfoliative cytology) by conventional method was done preoperatively for all. Those patients with abnormal pap smear or who had visibly unhealthy cervix, persistent inflammatory smears, discharge per vaginum not responding to medication or postcoital bleeding, underwent colposcopy. The colposcopic findings were recorded and scoring was done according to Reid's colposcopic index (RCI).

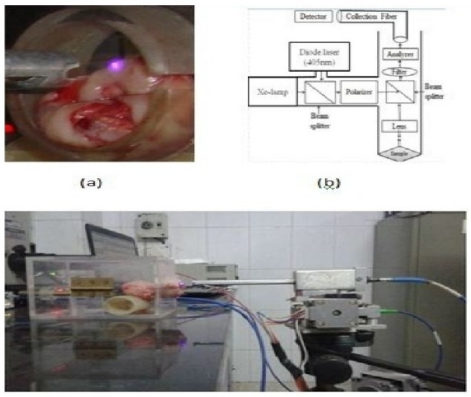
Principle of intrinsic fluorescence: The biological tissue has certain natural fluorophores with their particular absorption and fluorescence spectra. The optical properties of the diseased tissue differ from that of normal tissue due to change in concentration of contributing fluorophores. The fluorescence property of contributing fluorophore is affected significantly by absorbers and scatterers, therefore extraction of intrinsic fluorescence is done.

Contribution of FAD in cervical tissue: At 405 nm wavelength, FAD is the dominating fluorophore. Due to change in metabolism of dysplastic cells, FAD converts to its reduced form ($FADH_2$) within the cell, which is non fluorescent. This change in fluorescence is used to differentiate between normal and pre-invasive lesions.

Post operatively the cervix was examined to appreciate the fluorescence pattern of the cervical cells by an optical device based on the principle of intrinsic fluorescence spectroscopy designed and fabricated by IIT Kanpur. The device consisted of two light sources, a diode laser (405 nm) and a white light source (Xe-lamp). A high pass filter (450 nm) was used to eliminate the source effect. Circumferential examination of cervix was done by the optical device taking 12o'clock as reference point. The data from different

patterns of fluorescence of FAD molecules, for different segments was acquired and the average was taken. The peak intensity of emission spectra was analyzed and band area around it was calculated to differentiate between healthy and unhealthy tissue. Values were taken for both co-polarized (vv) light and cross-polarized (vh) light. The samples were then fixed in formalin and sent for histopathological evaluation. Figure 1(a) shows device tip projecting light on cervix; figure 1(b) shows schematic diagram; figure 1(c) the optical device set up beside operation theatre.

Fig 1 (a) Optical device tip projecting light on cervix (b) Schematic Diagram (c) the optical device set up beside OperationTheatre



Statistical tools employed: The statistical analysis was done using MedCalc software using receiver operative characteristic curve (ROC curve). Histopathology reports of the biopsies were taken as the gold standard. Sensitivities, specificities, receiver operating characteristic (ROC) curves, and areas under the ROC curves were calculated. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for Pap smear and colposcopy were calculated by drawing the contingency tables.

Results

Thirty one (19.01%) patients had pre-invasive lesion and 131 (80.29%) patients were either negative for intraepithelial lesion or malignancy (NILM) or had inflammatory smears in their pap smears. According to histopathology 41 (25.16 %) had some form of pre-invasive lesion and 121 (74.23%) were normal (table 1).

Pap smear findings were then correlated with histopathology. The sensitivity and specificity for pap smear was calculated as 47% and 90% respectively. The PPV was 62.5% and NPV was 83.2% (table 2).

Table 1: Distribution of cases according to pap smear and histopathology reports

Variable	Subjects (N=163)	Percentage (%)
Pap smear		
NILM	80	49.07
Inflammatory	51	31.28
LSIL*	21	12.88
HSIL**	10	6.13
Carcinoma	1	0.61
Total	163	100
Histopathology		
Normal	121	74.23
CIN I	29	17.79
CIN II	10	6.13
CIN III	2	1.22
Carcinoma	1	0.61
Total	163	100

*Low grade squamous intraepithelial lesion, **High grade squamous intraepithelial lesion

Table 2: Correlation of pap smear and histopathology reports (N=163)

Histopathology	Normal	CIN I	CIN II	CIN III	Carcinoma	Total
Pap smear						
NILM	61	19	0	0	0	80
Inflammatory	48	02	01	0	0	51
LSIL	09	07	04	01	0	21
HSIL	03	01	05	01	0	10
Carcinoma	0	0	0	0	01	01
Total	121	29	10	02	01	163

Colposcopy was done for 89 women and RCI score was calculated. The latter was correlated with histopathology reports. The sensitivity and specificity for colposcopy by RCI score was 92.3% and 86% respectively. The PPV was 83.7% and NPV was 93.4% at a cut off RCI score of 3 for detection of pre- invasive lesions (CIN I and above) (table 3).

Table 3: Correlation of RCI score with histopathology reports (N=89)

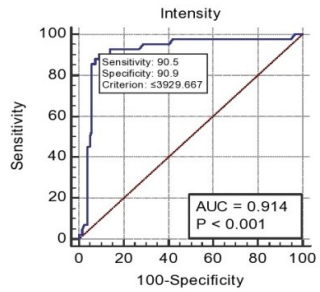
Histopathology	Normal	CIN I	CIN II	CIN III	Carcinoma	Total
RCI score						
0-2	43	02	01	0	0	46
3-4	07	20	07	0	0	34
5-8	0	04	02	02	01	09
Total	50	26	10	02	01	89

Figure 2 shows receiver operating characteristic (ROC) curve of intrinsic fluorescence spectroscopy using band area around peak intensity value with co-polarized (vv) light. The sensitivity and specificity for optical device using co-polarized light was found to be 90.5% and 90.9% respectively with area under curve of ROC analysis of 0.914 at band area 3929.66 (p value < 0.0001).

Figure 3 shows receiver operating characteristic (ROC) curve for intrinsic fluorescence spectroscopy using band area around peak intensity value with cross-polarized (vh) light. The sensitivity and specificity for optical device using cross polarized light was found to be 81.1% and 87.3%

respectively with area under curve of ROC analysis of 0.954 at band area 2962 (p value < 0.0001).

FIG 2 Receiver Operating Characteristic curve (ROC curve) for optical device using co-polarized



ROC curve

Variable	Intensity
Classification variable	Diagnosis

Sample size	163
Positive group [a]	42 (25.77%)
Negative group [b]	121 (74.23%)

[a] Diagnosis = 1
[b] Diagnosis = 0

Disease prevalence (%)	Unknown

Area under the ROC curve (AUC)

Area under the ROC curve (AUC)	0.914
Standard Error [a]	0.0295
95% Confidence interval [b]	0.860 to 0.952
z statistic	14.024
Significance level P (Area=0.5)	<0.0001

[a] DeLong et al., 1988
[b] Binomial exact

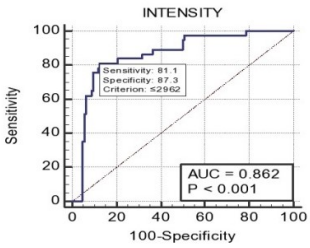
Youden index

Youden index J	0.8139
Associated criterion	≤3929.667
Sensitivity	90.48
Specificity	90.91

Criterion values and coordinates of the ROC curve

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	-LR
<608.9	0.00	0.0 - 8.4	100.00	97.0 - 100.0		1.00
≤608.9	2.38	0.06 - 12.6	100.00	97.0 - 100.0		0.98
≤1396.67	2.38	0.06 - 12.6	98.35	94.2 - 99.8	1.44	0.99
≤1617.09	4.76	0.6 - 16.2	98.35	94.2 - 99.8	2.88	0.97
≤1738.667	7.14	1.5 - 19.5	97.52	92.9 - 99.5	2.88	0.95
≤2184.88	7.14	1.5 - 19.5	95.87	90.6 - 98.6	1.73	0.97
≤3240.333	45.24	29.8 - 61.3	95.87	90.6 - 98.6	10.95	0.57
≤3327.66	45.24	29.8 - 61.3	95.04	89.5 - 98.2	9.12	0.58
≤3343.333	50.00	34.2 - 65.8	95.04	89.5 - 98.2	10.08	0.53
≤3344	52.38	36.4 - 68.0	94.21	88.4 - 97.6	9.05	0.51
≤3603.667	85.71	71.5 - 94.6	94.21	88.4 - 97.6	14.82	0.15
≤3787.76	85.71	71.5 - 94.6	92.56	86.3 - 96.5	11.52	0.15
≤3814.333	88.10	74.4 - 96.0	92.56	86.3 - 96.5	11.84	0.13
≤3923.333	88.10	74.4 - 96.0	90.91	84.3 - 95.4	9.69	0.13
≤3929.667	90.48	77.4 - 97.3	90.91	84.3 - 95.4	9.95	0.10
≤4320.55	90.48	77.4 - 97.3	85.95	78.5 - 91.6	6.44	0.11
≤4405.333	92.86	80.5 - 98.5	85.95	78.5 - 91.6	6.61	0.083
≤4983.667	92.86	80.5 - 98.5	72.73	63.9 - 80.4	3.40	0.098
≤5100.667	95.24	83.8 - 99.4	71.07	62.1 - 79.0	3.29	0.067
≤5800	95.24	83.8 - 99.4	59.50	50.2 - 68.3	2.35	0.080
≤5843.666	97.62	87.4 - 99.9	57.85	48.5 - 66.8	2.32	0.041
≤25775	97.62	87.4 - 99.9	4.96	1.8 - 10.5	1.03	0.48
≤25775.6673	100.00	91.6 - 100.0	3.31	0.9 - 8.2	1.03	0.00
≤41234	100.00	91.6 - 100.0	0.00	0.0 - 3.0	1.00	

FIG 3 Receiver Operating Characteristic curve (ROC curve) for optical device using cross-polarized (vh) light



ROC curve

Variable	INTENSITY
Classification variable	DIAGNOSIS

Sample size	163
Positive group [a]	37 (22.70%)
Negative group [b]	126 (77.30%)

[a] DIAGNOSIS = 1
[b] DIAGNOSIS = 0

Disease prevalence (%)	Unknown

Area under the ROC curve (AUC)

Area under the ROC curve (AUC)	0.862
Standard Error [a]	0.0347
95% Confidence interval [b]	0.799 to 0.911
z statistic	10.439
Significance level P (Area=0.5)	<0.0001

[a] DeLong et al., 1988
[b] Binomial exact

Youden index

Youden index J	0.6838
Associated criterion	≤2962
Sensitivity	81.08
Specificity	87.30

Criterion values and coordinates of the ROC curve

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	-LR
<104.666687	0.00	0.0 - 9.5	100.00	97.1 - 100.0		1.00
≤883.92	0.00	0.0 - 9.5	95.24	89.9 - 98.2	0.00	1.05
≤1398	35.14	20.2 - 52.5	95.24	89.9 - 98.2	7.38	0.68
≤1407	35.14	20.2 - 52.5	94.44	88.9 - 97.7	6.32	0.69
≤1552.333333	51.35	34.4 - 68.1	94.44	88.9 - 97.7	9.24	0.52
≤1608	51.35	34.4 - 68.1	93.65	87.9 - 97.2	8.09	0.52
≤1878	62.16	44.8 - 77.5	93.65	87.9 - 97.2	9.79	0.40
≤2176.66	62.16	44.8 - 77.5	91.27	84.9 - 95.6	7.12	0.41
≤2296.87	64.86	47.5 - 79.8	91.27	84.9 - 95.6	7.43	0.38
≤2340.04	64.86	47.5 - 79.8	90.48	84.0 - 95.0	6.81	0.39
≤2599	75.68	58.8 - 88.2	90.48	84.0 - 95.0	7.95	0.27
≤2878	75.68	58.8 - 88.2	88.10	81.1 - 93.2	6.36	0.28
≤2890	78.38	61.8 - 90.2	88.10	81.1 - 93.2	6.58	0.25
≤2901.01	78.38	61.8 - 90.2	87.30	80.2 - 92.6	6.17	0.25
≤2962	81.08	64.8 - 92.0	87.30	80.2 - 92.6	6.39	0.22
≤3177.62	81.08	64.8 - 92.0	79.37	71.2 - 86.1	3.93	0.24
≤3180.333333	83.78	68.0 - 93.8	79.37	71.2 - 86.1	4.06	0.20
≤3488.98	83.78	68.0 - 93.8	68.25	59.4 - 76.3	2.64	0.24
≤3499.333333	86.49	71.2 - 95.5	68.25	59.4 - 76.3	2.72	0.20
≤3609.67	86.49	71.2 - 95.5	63.49	54.4 - 71.9	2.37	0.21
≤3622	89.19	74.6 - 97.0	63.49	54.4 - 71.9	2.44	0.17
≤3879.94	89.19	74.6 - 97.0	50.00	41.0 - 59.0	1.78	0.22
≤3948.333333	94.59	81.8 - 99.3	50.00	41.0 - 59.0	1.89	0.11
≤3948.86	94.59	81.8 - 99.3	49.21	40.2 - 58.3	1.86	0.11
≤3976.666667	97.30	85.8 - 99.9	49.21	40.2 - 58.3	1.92	0.055
≤5845.666667	97.30	85.8 - 99.9	21.43	14.6 - 29.6	1.24	0.13
≤5855.666667	100.00	90.5 - 100.0	21.43	14.6 - 29.6	1.27	0.00
≤25775.6673	100.00	90.5 - 100.0	0.00	0.0 - 2.9	1.00	

Discussion

Pap smear shows wide range of sensitivity and specificity as seen in various cross sectional studies done in India and abroad. Sensitivity varied from 44% to 75%, and the

specificity varied between 91% and 98.9% [8, 21, 22]. In the systemic review done by Nanda et al, pap smear sensitivity ranged from 30% to 87% and specificity ranged from 86% to 100% [6]. According to cochrane review of forty studies on over 140,000 women, sensitivity and specificity for pap smear was found to be 62.5% and 96.6% respectively [23]. In our study, sensitivity of pap smear was found to be 47% while specificity was 90% which is comparable to results in the above mentioned studies.

Reid's colposcopic index (RCI) score was then correlated with histopathology. The sensitivity was 92.3% which was higher than the specificity (86.0%). PPV and NPV were found to be 83.7% and 93.47% respectively at a cut off Reid's score 3, for detecting pre - invasive lesions. Studies done in Indian population by Durdi et al and Kushwah, also demonstrate the accuracy of colposcopy similar to that found in our study. Durdi et al studied 268 women and found the sensitivity as 88.5% which was greater than specificity (86.2%), Similarly Kushwaha conducted the study on 80 women and found sensitivity & specificity as 94.44% & 91.48% respectively [24, 25].

According to a study done by Bhalerao et al incidence of invasive carcinoma was 3/200 = 0.01% and was contributed to premalignant condition CIN II, III. Acetowhite was the most common colposcopic finding and the most common biopsy result in women with inflammatory smear was chronic cervicitis. The sensitivity of Pap smear was 91.33% which was high as they had recruited women with unhealthy cervices [26]. A meta-analysis by Olaniyan in 2002, on validity of colposcopy in early diagnosis of early cancer cervix showed a very high sensitivity ranging from 87-99% [27]. The results in our study are consistent with the above studies.

The hypothesis that NADH and FAD are the source of fluorescence in abnormal cervical cells is supported by many studies [17, 18]. Cantor SB et al studied the accuracy of optical spectroscopy for detection of CIN and calculated the sensitivity of 100% and specificity 71% [12]. Similar results were demonstrated by Jing et al where they studied native fluorescence on cervical tissue and found sensitivity and specificity to be 100% and 91% respectively [28].

Since intrinsic fluorescence is free from absorption and scattering effects, it provides more precise information about biochemical changes [29, 30]. Scientific literature shows that fluorescence spectroscopy has a potential of detecting premalignant lesions in different biological tissues such as breast, lung and oral cavity [14, 31, 32]. The optical device used in our study analysed respective fluorescence spectra from tissues using co- and cross polarized lights after extraction of the intrinsic fluorescence. The sensitivity and specificity for optical device using co-polarized light was found to be 90.5% and 90.9% whereas the sensitivity and specificity for cross polarized light was found to be 81.1% and 87.3% respectively, which was lower than the co-polarized light. The sensitivity, specificity and area under the ROC curve were better with the co-polarized light. These results add to substantial evidence in previous studies [28,33,34].

Conclusion

Due to the high sensitivity and specificity, fluorescence spectroscopy is an important area of research for being used as screening purpose in medical field. It has reasonable efficacy, is fast and gives quick results. In addition, while the subjective nature of commonly used screening methods lead to inter and intra observer variations, the fluorescence spectroscopy based method gives an objective picture. The portable optical device based on this principle has the potential to be an excellent screening tool which can provide see-to-treat protocol and early intervention for pre invasive lesions.

Future improvements: 1. To make the device biocompatible and handy for community use. 2. To differentiate between low grade and high grade pre cancerous lesions 3. Presently this technology is limited to native autofluorophores but further work can be done on using exogenous agents which can probe the particular molecules specific for a cancer cell.

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Conflict of interest: None. **Disclaimer:** Nil.

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