Cancer Diagnostic Nano-Probe Based on Raman Spectroscopy:

In the area of cancer diagnostics, Dr. Maiti developed functionalized nano-particle probes for ultrasensitive detection of various human cancer biomarkers viz., cervical, breast, lung and prostate, glioma using Raman scattering (surface enhanced Raman scattering: SERS) and Imaging as a diagnostic modality. **Two major inventions have been emphasized as follows**:

A. Diagnostic Spectro-Cytology for Differential Recognition of Cervical Cancer Lesions by Label-free Surface Enhanced Raman Fingerprints and Artificial Intelligence (AI)

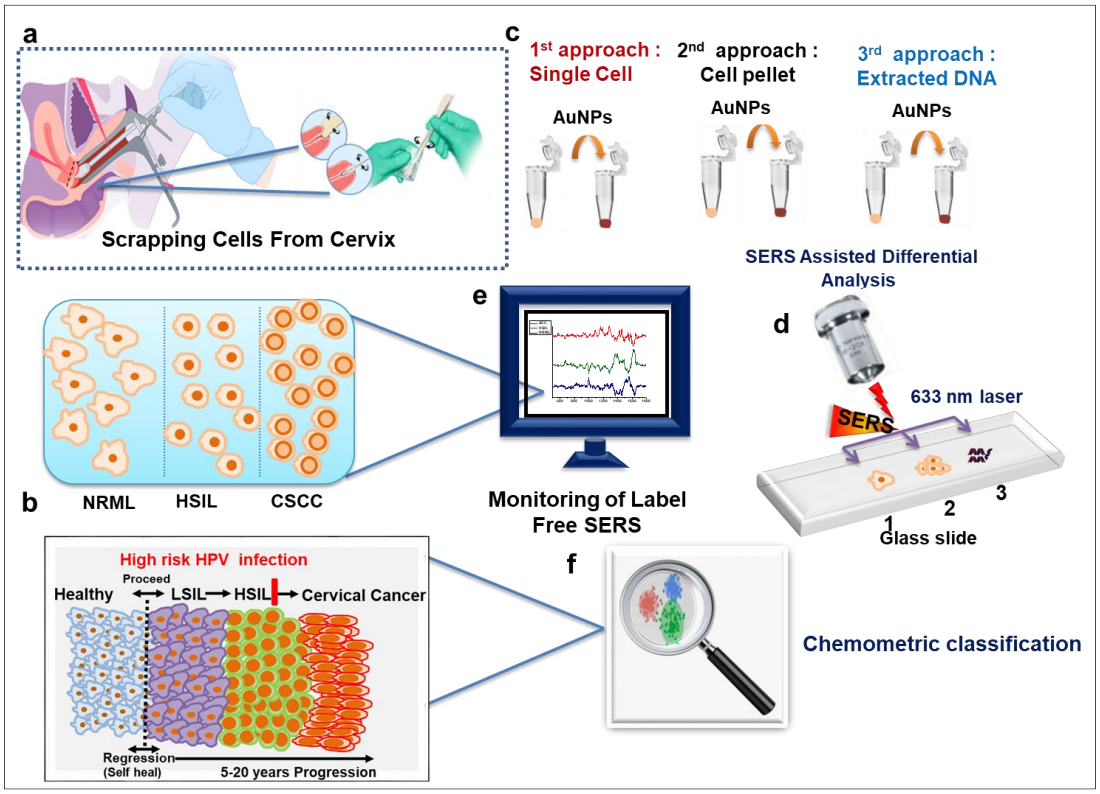
Background of the diagnostic Spectro-cytology for Cervical Cancer

Cancer of the uterine cervix is one of the most common cancers among women worldwide and the second most prevalent cancers among women in India. It is considered as a preventable cancer as cervix is an easily amenable organ and occurrence of a heterogeneous spectrum of epithelial abnormalities (precancerous lesions) 10-15 years prior to the occurrence of invasive cancer has been well established. The significance of Pap smear test for the detection and eradication of these precancerous lesions of cervix has been well documented. Based on this, systematically organized screening programmes for cervical cancer has been implemented in many of the developed countries. The role of persistent infection with high-risk HPV in cervical carcinogenesis is now well established and prophylactic vaccine against two of the high-risk HPVs are currently available. Considering the several other high-risk strains of this virus prevalent among the women, vaccine plus Pap smear test and HPV DNA test is now advocated for the effective control this disease. However, in India and many other low resource countries none of these measures have been implemented so far and cervical cancer continues to take extraordinary toll on the lives of our women. The major impediment for implementing screening programme by Pap smear is the lack of trained cytologists for microscopic analysis of the Pap smears of the eligible women of the community. So, there is an urgent need for alternative cost effective and reliable method of screening without the requirement of highly trained cytologists.

Non-invasive diagnosis of malignancies has been well-studied by optical spectroscopic methods, mostly by Fourier-transform infrared spectroscopy, Raman, fluorescence etc. and out of which Raman spectroscopy offers some distinctive benefits over other techniques. Among various types of Raman scattering techniques, surface enhanced Raman scattering (SERS) received huge acceptance in the research community as it is a powerful technique which allows the ultrasensitive detection of analytes with low concentration through the amplified signals

around 10^8–10^{14} folds by the excitation of surface plasmons rendered by metallic nanoparticles such as gold nanoparticles (AuNPs) as SERS substrate.

The current diagnostic practices in clinic, i.e., Pap test and HPV DNA identification suffer drawbacks like less sensitivity, subjective nature and is time consuming. Herein, an efficient diagnostic platform has been developed to identify precancerous lesions (high-grade squamous intraepithelial lesion (HSIL) and cervical squamous cell carcinoma (CSCC) in the cells exfoliated from uterine cervix using a differential Raman spectral pattern based on label free surface enhanced Raman scattering (SERS) technique. A differential spectral fingerprint for the prediction of normal NRML, HSIL and CSCC has been generated by comparing three different approaches i.e., single-cell, cell-pellet and extracted DNA. The tunable plasmonic properties of the gold nanoparticles as the SERS substrate favored the increment of Raman intensity in minimal time in an ultrasensitive manner. The ratio of SERS signal intensities between 1270 /1370 cm^{-1} in single cell and 956 /1022 cm^{-1} in cell pellet exhibiting the signature identity for Amide III/Nucleobases and carotenoid/glycogen respectively seemed proficient for establishing the empirical discrimination. **A**ll the spectral invention was subjected to chemometrics (Machine learning algorithm) including Support Vector Machine (SVM) which furnished an average diagnostic accuracy of **93.84 %, 74.26 % and 92.21 %** for single cell, cell pellet and extracted DNA respectively.



Schematic illustration of experimental design for differentiating three grades viz. normal (NRML), high grade intraepithelial lesion (HSIL), cervical squamous cell carcinoma (CSCC) using SERS., a) Scrapping cells from the cervix using cytobrush, b) progression pattern of cervical cancer c) Set 1: single cell, Set 2: cell pellet, Set 3: extracted DNA (mixed with AuNPs), d) independent SERS analysis

of 1) single cell,2) cell pellet, 3) extracted DNA in glass slide, d) empirical signal monitoring of the three grades f) chemometric analysis.

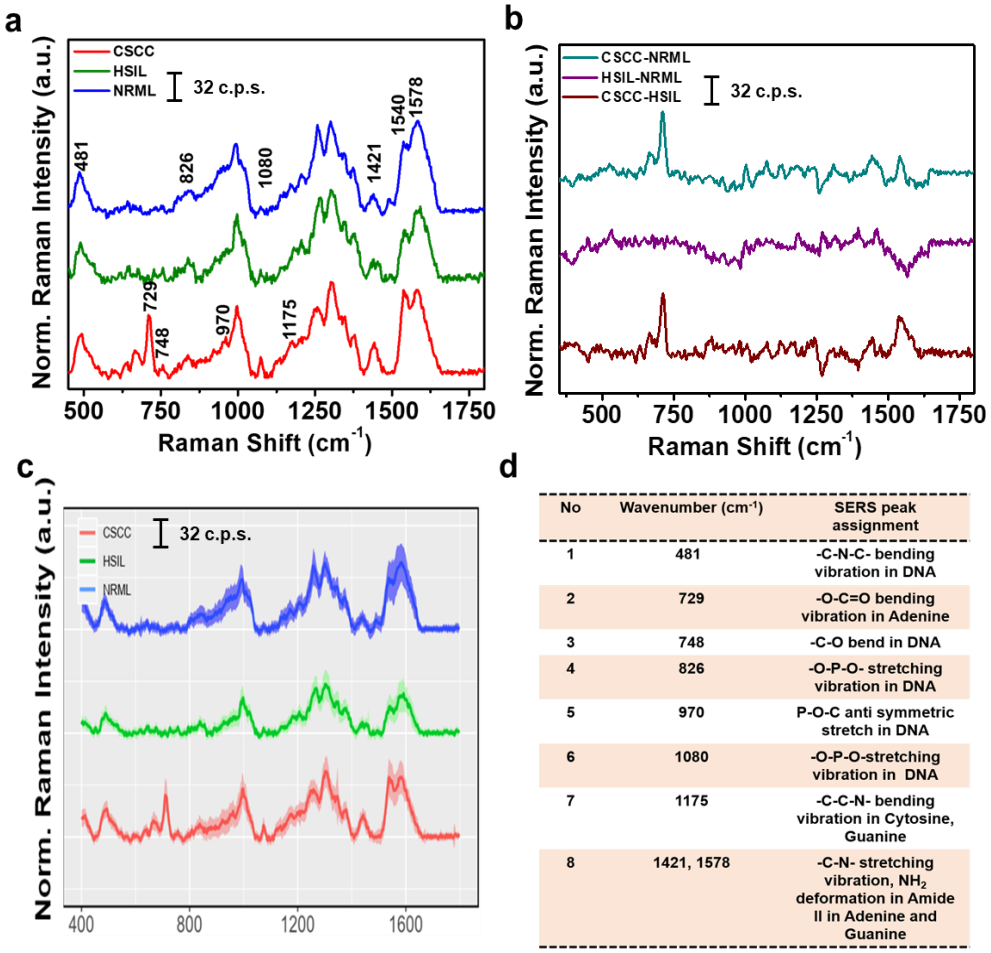
Technical Details & Results

Label-free SERS Spectral Analysis

Pathologically confirmed cervical smears of major three grades i.e., NRML, HSIL and CSCC were collected and processed using liquid based cytology procedure. A smear button was prepared, which concentrated diagnostically relevant cells upon incubation with AuNPs (as Raman signal enhancer) by removing the mucus and blood by repeated density gradient centrifugation. The fingerprint spectral information was extracted mostly in the nuclear region of the single cells as the variation arising from the cytoplasmic region can be minimized. Since the analysis involved addition of AuNPs, Pap-stained slides were subjected to de-staining followed by SERS analysis to select the diagnostically relevant cells. Initially, individual SERS fingerprint was evaluated empirically followed by chemometric statistical analysis to group the three classes. The mean spectra accumulated from the average of the collected spectrum excluding the outliers of each group were normalized to its highest peak (Amide II at 1550 cm^{-1}) in which clear spectral variations were observed. The analysis in exfoliated single cell samples, NRML, HSIL and CSCC showed distinctive peaks correlating to the cell line signals. Distinct Raman peak at 481cm^{-1} associated with -C-N-C bending vibration of DNA was found to be increased in HSIL and CSCC samples which showed an indication of high nuclear content. The -O-C=O- bending vibration of amino acid tryptophan corresponding to 573 cm^{-1} was prominent in HSIL and CSCC samples displaying the presence of high protein content which may be mostly from the histone protein and nuclear regulatory proteins inside the nucleus. The -O-C=O- bending vibration peak at 643 and 666 cm^{-1} are indicative to tyrosine, thymine and guanine ring vibrations present in all grades whereas adenine ring vibration at 729 cm^{-1} identified prominently in the abnormal grades i.e., HSIL and CSCC predicting the increase in nuclear elements. On the other hand, O-P-O stretching at 826 cm^{-1} and 1080 cm^{-1} favored the indirect existence of nucleic acid. The O-P-O stretching at 826 cm^{-1} showed the increased nuclear content in abnormal samples. Interestingly the peak at 826 cm^{-1} in HSIL and CSCC samples is identified as a shifted position in comparison to normal samples which showed a prominent peak at 850 cm^{-1}. Another distinguishing factor between the three groups were the presence of -C-O-C- stretching of the amino acid proline. It has been proven that proline rich tyrosine kinase 2 (Pyk2) plays an important role in tumor progression in various human cancers. Pyk2 is a non-receptor tyrosine kinase which controls tumor survival, its proliferation, migration, invasion properties, metastasis and resistance to chemotherapy. In addition, there exists a correlation of a biomarker known as c-myc over expression distinct in cervical cancer which increases proline biosynthesis from glutamine and is a prognostic marker useful in guiding treatment decisions in cervical cancer. The increase of carotenoid signal at 956 cm^{-1} and 1165 cm^{-1} in single cells might be due to the reason that cancer cells tend to accumulate carotenoids to resist damage. Amide III signal from proteins at 1262 cm^{-1} in NRML is shifted

in HSIL and CSCC samples to 1270 cm^{-1}. Similarly, amide II signal arising at 1560 cm^{-1} from the protein counterparts inside the nucleus showed a clear shift of around 10 nm between normal and abnormal samples. Interestingly, the ratio between 1270 and -O-C=O symmetric stretching at 1373 cm^{-1} were well resolved for the prediction of abnormality from the mean spectra. The ratio value was found to be 1.55, 1.2 and 1.02 for NRML, HSIL and CSCC respectively in single cells. The ratio is decreasing because the peak corresponding to 1373 cm^{-1} is increasing in the abnormal samples. The variations existing between the three groups were acquired by subtracting the mean spectra of NRML from CSCC, NRML from HSIL and HSIL from CSCC. The positive peaks in the difference spectra obtained showed the presence of bio-molecular activity and negative peaks showed the absence of the same.

In the progression of Raman fingerprinting obtained from single cell and cell pellet analysis for the differential diagnosis of cervical precancerous and cancerous lesions, cellular DNA was extracted to re-investigate nucleotide profiling. Increase in DNA content was evident between the groups when compared with normal counterparts. Majority of the SERS peaks obtained from extracted DNA correlated with the peaks obtained from the single cell and cell pellet. The specific peaks at 729 cm^{-1} corresponded to -O-C=O- adenine ring vibration, 826 and 1080 cm^{-1} related to the O-P-O stretching vibration in DNA, 1172 cm^{-1} attributed to -C-C-N- bending vibration in cytosine and guanine, 1421 and 1578 cm^{-1} were related to -C-N- stretching vibration and NH_2 deformation in Amide II in adenine and guanine. Difference spectra were assessed by subtracting NRML from CSCC, NRML from HSIL and HSIL from CSCC mean spectra from the DNA samples (Figure 1).



No	Wavenumber (cm^{-1})	SERS peak assignment
1	481	-C-N-C- bending vibration in DNA
2	729	-O-C=O bending vibration in Adenine
3	748	-C-O bend in DNA
4	826	-O-P-O- stretching vibration in DNA
5	970	P-O-C anti symmetric stretch in DNA
6	1080	-O-P-O-stretching vibration in DNA
7	1175	-C-C-N- bending vibration in Cytosine, Guanine
8	1421, 1578	-C-N- stretching vibration, NH_2 deformation in Amide II in Adenine and Guanine

Figure:1 *a) Mean SERS spectra [Laser power density 3-7 mW power density] b) the difference spectrum c) the standard deviation and d) tentative SERS peak assignments from cervical clinical DNA samples; NRML, HSIL and CSCC.*

Chemometric evaluation of differential SERS spectra of NRML, HSIL and CSCC by PCA, LDA and SVM

A huge spectral data set in all the three groups NRML, HSIL and CSCC were discriminated by chemometric modelling and prediction within the dataset employed for extracting information comprising complex data set from a chemical or biological source based on multivariate, mathematical or computational models. Initially, adopted chemometric analysis named PCA & LDA to classify them using MATLAB software. A predictive classification was obtained using PCA analysis in single cell, cell pellet and extracted DNA respectively. In the course of gradual improvement of prediction accuracy, SVM analysis was attempted by randomly selecting 75 % of the spectra as the train set and rest 25 % were used as the test set. The SVM analysis was repeated with 500 different random samples and measured the average prediction accuracy. **The accuracy was found to be 93.84 % for single cell, 74.26 % for cell pellet and 92.21 % for extracted DNA** with 0.73 %, 5.04 % and 3.84 % standard deviation respectively (Figure 2). Thus, based on the created reference spectral module an unknown sample can be predicted. The percentage of prediction accuracy was generated along with ROC curve (**Figure**

2). ROC curve is a graphical plot which shows the diagnostic ability of a classifier system by varying the discrimination threshold. At different threshold setting, the ROC curve is plotted by true positive rate against false positive rate. True positive is termed sensitivity and false negative is termed 1-specificity. The accuracy of the analysis depends on how good the test separates the group got tested into those with and without the disease. The ROC curve of single cell, cell pellet and extracted DNA showed that SVM is an incremental diagnostic model for classifying the groups **(Figure 2g, h, i).** The sensitivity and prediction accuracy of the technique was calculated (**Table 1**). Out of the performed methods, SVM showed a prominent classification between the grades.

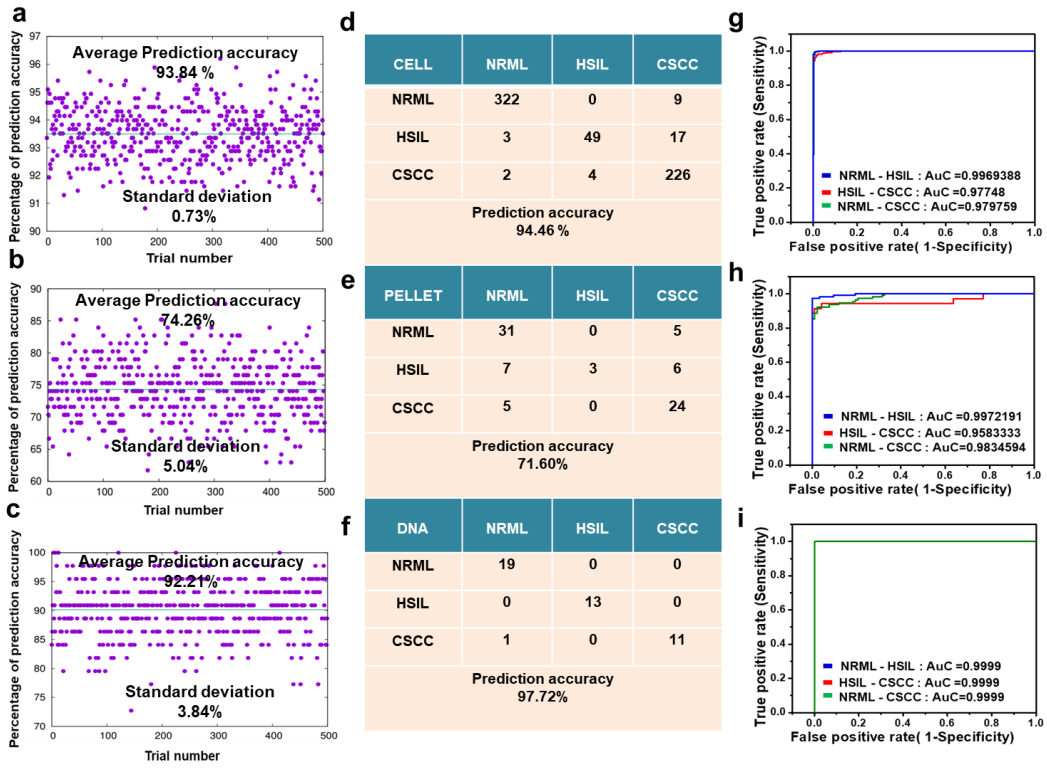


Figure 2. *Percentage of average prediction accuracy chart in a) single cell, b) cell pellet and c) extracted DNA, Classification based on SVM analysis of d) single cell, e) cell pellet and f) extracted DNA, SVM ROC curve in g) cell, h) pellet, i) DNA*

Table 1. Sensitivity and prediction accuracy of SERS-based cervical precancerous lesions detection

	Sample	Total	True Prediction	False Prediction	Prediction Accuracy
Single Cell	**NRML**	**331**	**322/331**	**9/331**	**94.46%±0.73**
	HSIL	**69**	**49/69**	**20/69**	
	CSCC	**232**	**226/232**	**6/232**	
Cell Pellet	**NRML**	**36**	**31/36**	**5/36**	**71.60%± 5.04**
	HSIL	**16**	**3/16**	**13/25**	

	CSCC	**29**	**24/29**	**5/29**	
Extracted DNA	**NRML**	**19**	**19/19**	**0/19**	**97.72%± 3.84**
	HSIL	**13**	**13/13**	**0/13**	
	CSCC	**12**	**11/12**	**1/12**	

Investigation of cytopathological evaluation of cervical exfoliated cells by Papanicolaou (Pap) staining:

Conventional analysis like PAP smear were performed to correctly identify the desired cells for SERS based analysis and subsequent validation of the technique. Normally, cervical cancer is diagnosed using cytopathology analysis commonly known as Pap smear test where single cells are tested. As our sample source of interest was exfoliated cells, Pap staining was performed to identify the pathologically relevant cells. In order to get a clear discrimination between normal vs abnormal exfoliated cells, both bright field images from confocal Raman microscope and Pap staining of NRML, HSIL and CSCC were evaluated for morphological analysis. All the abnormal samples were further confirmed by colposcopic biopsy. In Pap staining, the superficial NRML cells were stained pink with pyknotic nucleus, intermediate cells-stained light blue to green colour where as in HSIL, the enlarged nucleus reflecting high DNA content with minimal cytoplasm showed purple colour nucleus. In CSCC, the cells started to show invasive nature with a slender shape formation to the nucleus. To correctly identify the desired classes of cells, initially the Pap staining was performed to mark the cell position and the same cells were subjected to SERS analysis. If only normal cells are present, the Pap tests predict as normal but in case of abnormal or atypical cells, it turned out as atypical squamous cells of undetermined significance (ASCUS), precancerous squamous intraepithelial lesion, atypical glandular cells, squamous cell cancer or adenocarcinoma cells etc. The primarily focus of the work was to establish a successful discrimination modality of NRML, HSIL and CSCC cells by SERS analysis after pathologically confirmed by Pap test.
The PCR product obtained was indicative of oncogenic HPV types 16, 18, 31, 33, 35, 45, 52 and 58.

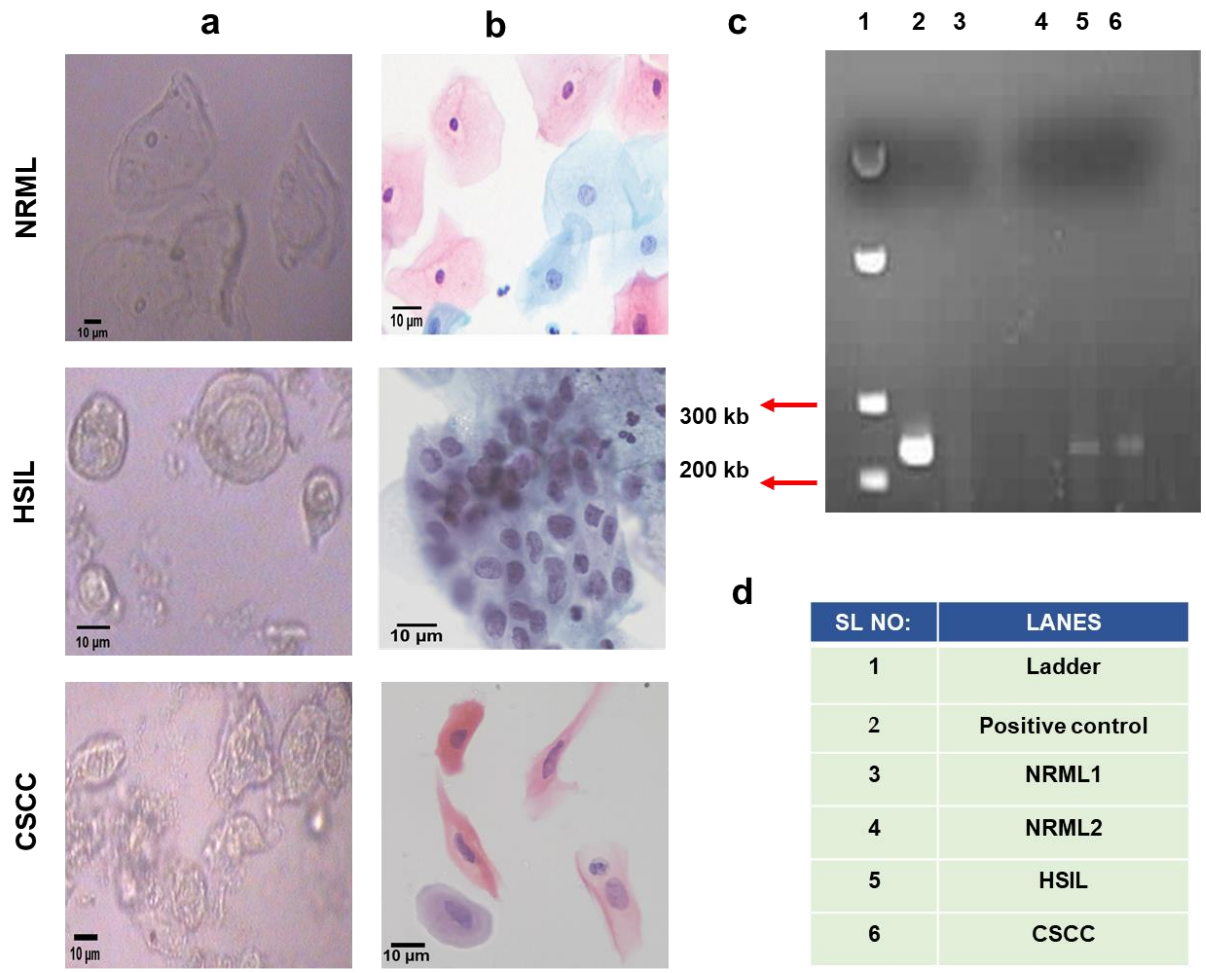


Figure 3. *a) Bright field and b) Pap stained images of NRML, HSIL and CSCC exfoliated cells, Scale bar corresponds to 10µm, c) HPV PCR of Clinical DNA samples, d) Sample loading order of HPV PCR Ladder (1), Positive control (2), NRML1 (3), NRML2 (4), HSIL(5), CSCC(6).*

Major Advantages and Conclusion

In summary, a label free ultrasensitive Raman fingerprint has been benchmarked to generate a differential mapping for the identification of major three grades of cervical exfoliated cells i.e., normal (NRML), intermediate (HSIL) and squamous cell carcinoma (CSCC) of the cervix by employing a strategic platform based on SERS. A clear identification of SERS fingerprint of the well resolved biomolecular variations ranging from nucleic acid, amino acids, protein backbone were established between NRML, HSIL and CSCC of clinical samples. The results indicated the prevalence of nucleobases adenine, guanine, cytosine, phosphate backbone and amino acids tryptophan, phenyl alanine and proline which are the major metabolites evolved during tumorigenesis. Further spectral differentiation was validated by statistical analysis which included chemometric interpretations mainly by PCA, LDA and

SVM. This is the first representation of SERS spectroscopic analysis in exfoliated cell samples. Moreover, the SERS based spectro-cytology was found to be minimally invasive and more sensitive than those employing serum or plasma as exfoliated cells represents a better source of sample for analyzing progression of cervical cancer. As immunostaining is a time consuming and skilled cytotechnologists are required for correct evaluation and HPV PCR causes nonspecific amplification of abnormal samples irrespective of its grades and expensive, SERS is an accurate, simple and reliable technique which can differentiate normal, HSIL and cancerous CSCC samples through its differential spectra and successfully predicted through chemomertic interpretation. Taken together, the present strategy represents an accurate, simple and reliable method for the differential diagnosis of cervical cancer which might serve as a clinical detection technique in the near future.

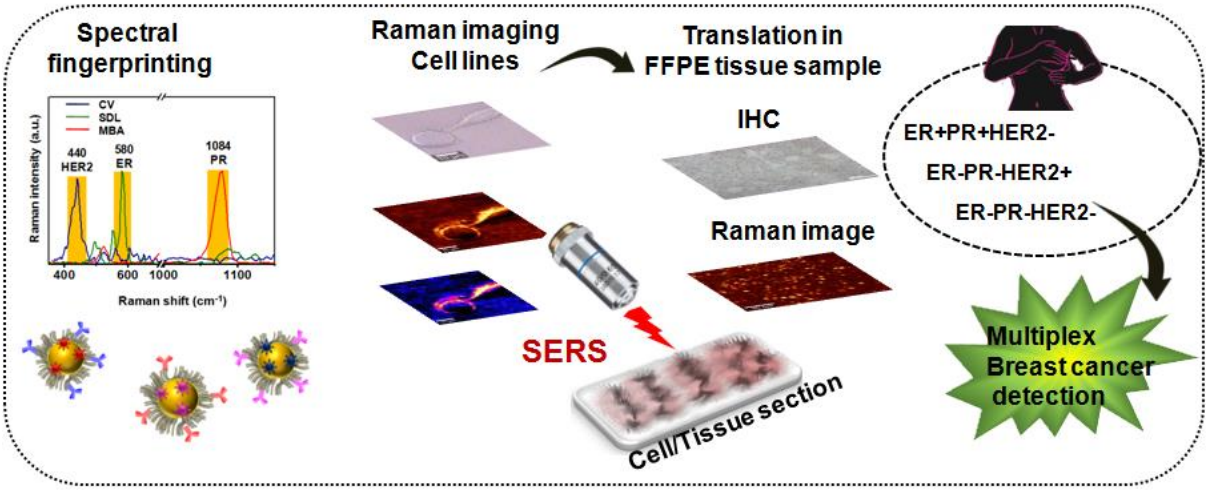
B. Clinically Relevant Multiplex Detection of Breast Cancer Biomarkers in Tissue Samples by SERS-Nanotags as Diagnostic Kit

In the area of breast cancer, Dr. Maiti developed diagnostic screening kit for concomitant detection of multiple breast cancer biomarkers in breast tissue samples using antibody conjugated SERS-nanotags. The kit can be used for real-time detection of the biomarkers, as and when the sample tissue is extracted from source. Hence, this kit has immense potential to develop immediate treatment strategies in heterogeneous breast cancer cases.

Background of the diagnostic kit (SERS-Nanotags) for Multiplex detection of Breast Cancer Biomarkers

Breast cancer is the most common cancer among women. Hormone receptors including Estrogen receptor (ER) and Progesterone receptor (PR) status are key biomolecules in breast cancer. Over-expression of HER2/Neu gene is associated breast cancer patient's prognosis and therapy and Ki67 is a proliferative marker. ER, PR, HER2 and Ki67 panel is essential in an estimation process of breast cancer prognosis which plays a significant role in treatment choice for breast cancer worldwide. Multiplexed detection is an attractive strategy in cancer diagnosis where multiple biomarkers can be evaluated simultaneously at a particular time. Recognition of pertinent biomarkers in heterogenous breast cancer facilitates clinicians in improving treatment strategies. Although current gold standard immunohistochemistry is capable of detecting single biomarker at a time, its subjective nature, inability of multiplexing and time-consuming nature makes it a hurdle leading to delayed results. In the current strategy, an alternative multiplex-detection technique has been developed for conventional IHC and FISH analysis by sequential addition of diagnostic SERS-nanoprobes with unique Raman reporters having multiplexing capability for the ultrasensitive and rapid detection of differential biomarkers, ER, PR and HER2 in breast

cancer. Initially the SERS-tags were validated in differential biomarker expressed cell line models and later the study was extended for diagnosis in clinically confirmed retrospective formalin fixed paraffin embedded (FFPE) breast cancer tissue samples in single-plex, duplex as well triplex manner. Multiplexing SERS was compared critically in terms of time required for the analysis and found to be an excellent technique which minimizes the processing and experiment time to around 5-6 hr unlike IHC for covering a 5 x 5 mm tissue area. Additionally, HER2 biomarker grading which is executed conventionally using time consuming IHC and expensive FISH analysis was also proved by SERS spectral analysis showing the potential of SERS to be applied in clinics. **Simultaneous detection of these biomarkers enabled to achieve a sensitivity of 95% and a specificity of 92% for single-plex analysis, 88% and 85% for duplex analysis, 75% and 67% for triplex analysis r**espectively. Combined diagnosis of these biomarkers enabled SERS-tags based detection to be turned out as an accurate, inexpensive, reliable and facile technique which can simultaneously identify and the biomarkers variations in different breast cancer subtypes in a semi-quantitative manner.



Technical Details and Results

Fabrication of SERS-Nanotags (Diagnostic kit)

Gold nanoparticles (AuNPs) were chosen as the SERS substrate with around 40-45 nm size for the design of multiplexed SERS-nanotags for the differential recognition of clinically relevant biomarkers viz. ER, PR and HER2 in breast cancer as this size reported to have the best SERS activity so far. SERS nanotags were fabricated by tagging three representative Raman reporter dyes having precise non-overlapping signature fingerprint peaks representative of each biomarker (ER, PR and Her2). The Raman reporters selected for the multiplexing analysis were commercially procured crystal violet (CV), 4-mercapto benzoic acid (MBA) and in-house

synthesized squaraine based Raman reporter, Squaraine Di-lipoic acid (SDL) having distinctive non-overlapping multiplexing Raman peaks at 440, 1084 and 580 cm^{-1} respectively. For facilitating stability and biocompatibility of the SERS nanoprobe, PEG coating was performed in the nanoparticle incorporated Raman reporter which also reduces toxicity of the Raman reporter as well as renders functional groups for antibody conjugation as well. Stickiness nature of nanoparticles to the vial was a limiting factor during the preparation of SERS tags leading to a reduced signal intensity due to particle loss which was efficiently tackled by the use of tween20. Finally, the corresponding antibodies for ER, PR and HER2 biomarkers were conjugated to the pegylated nanotags by modified version of the standard protocol for antibody conjugation, wherein we used Sulfo-NHS in MES buffer instead of NHS for better reactivity. The UV-Vis absorbance exhibited a 260 nm protein absorption peak with a small 1-2 nm shift from the 530 nm plasmon peak confirming successful antibody conjugation. SDS-PAGE gel analysis by silver staining also confirmed efficient antibody conjugation where the conjugated nanotag displayed a slight upshift as shown along with pure antibody (Figure 1a).

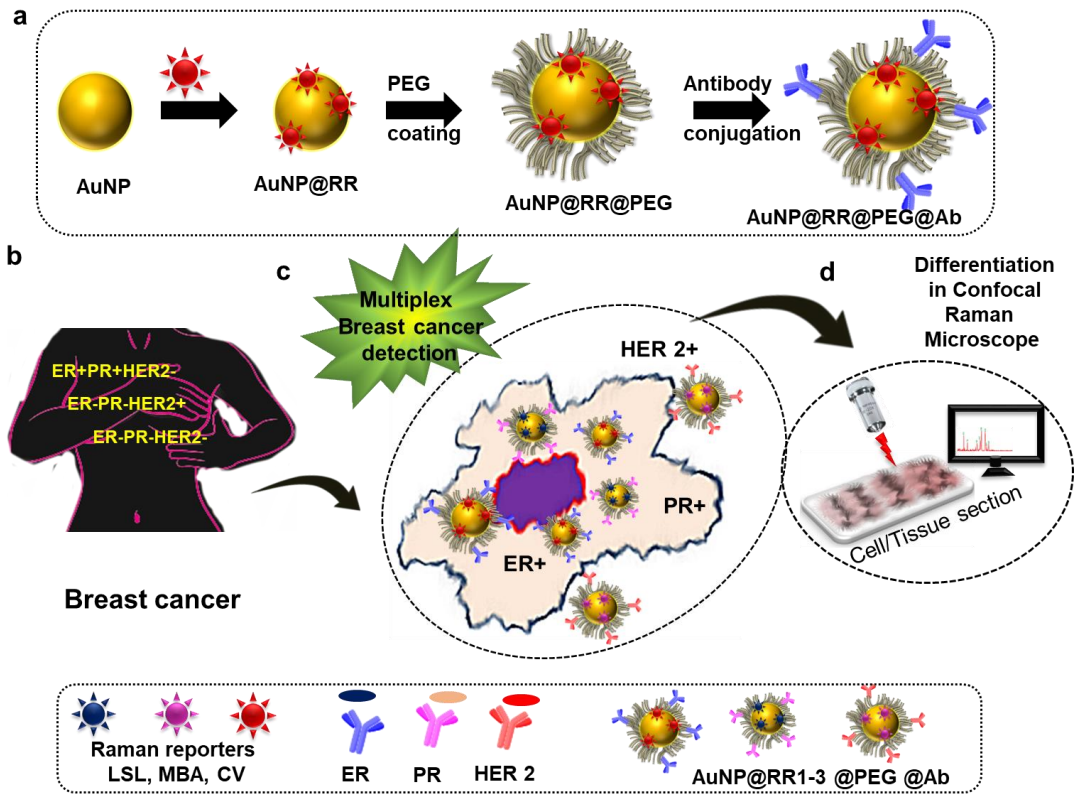


Figure 1: Illustration for experimental design for differentiating the clinically relevant triple biomarkers, ER, PR and HER2. a) Preparation strategy for the multiplexed SERS-tags by using AuNPs based substrate, b) biomarker detection of ER, PR and HER2 biomarker in three cell lines (MCF7, MDA-MB-231and SK-BR-3) having differential expression using confocal Raman microscope c) representative design for detection of biomarkers in cells/tissues by SERS-tags, d) SERS analysis and mapping in retrospective paraffin embedded differential biomarker expressed breast tissue samples after antigen retrieval using confocal microRaman system.

Multiplex Breast Cancer biomarker detection using SERS-nanotags

The SERS based detection in paraffin removed tissues was compared with the gold standard conventional immunohistochemistry (IHC) analysis. Three sets of tissue biomarker analysis were carried out viz., single plex analysis for either ER, PR or HER2, duplex analysis with a combination of any two out of this and triplex analysis with all the three biomarkers. During SERS analysis of tissue samples, sequential addition of nanotags as well as SERS nanotag cocktails were experimented out of which sequential addition showed impressive efficacy in terms of SERS signals intensity and accuracy. Similarly, MES buffer wash to remove excess unbound SERS tags was also found to be beneficial over PBST wash in terms of signal strength. IgG isotype antibody conjugated to SERS nanoparticles with DTNB as the Raman reporter was used as a control to achieve a ratiometric calculation of the prevalence of biomarker expression in the samples and also to eliminate the false positive results arose due to the nonspecific binding of the nanoparticles to the tissue samples.

Duplex analysis for tissue biomarkers

Even though a few kits based methods are there, dual biomarker detection is a challenging thing to attain in IHC, especially for breast cancer biomarkers. Using the current SERS based platform, various combinations of duplex biomarker analysis were performed in breast tissue samples having differential biomarker expression status. In an IHC confirmed ER^+ $HER2^-$ breast cancer tissue samples, sequential addition of AuNP@SDL@PEG@anti-ER followed by AuNP@CV@PEG@anti-HER2, led to the generation of only 580 cm^{-1} peaks from SDL specifying the overexpression of ER biomarker. The lack of noteworthy peak from 440 cm^{-1} of CV confirmed the minimal expression of HER2 in the tissue sample (Figure 2).

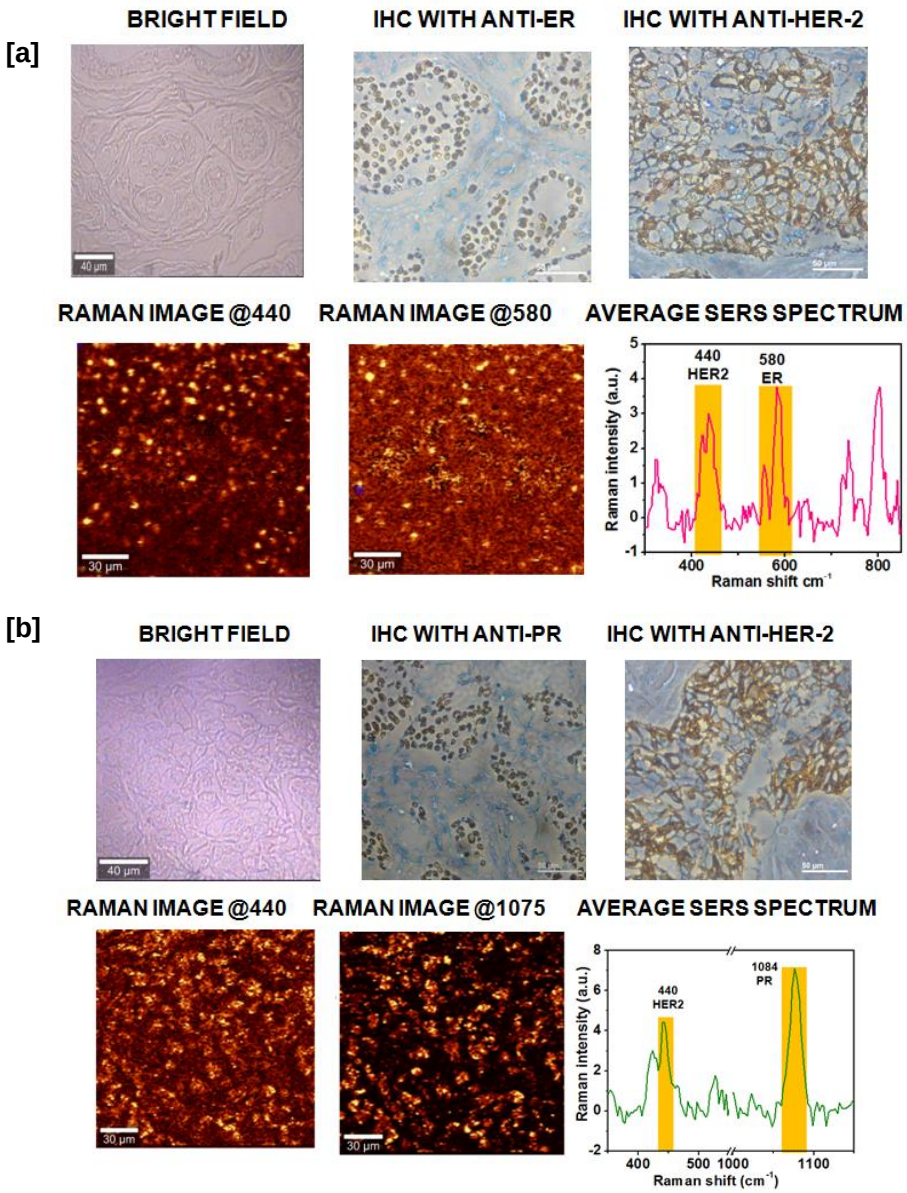


Figure 2: *Bright field images, Immunohistochemistry analysis and Raman imaging of a) ER^+HER2^+tissue using AuNP@SDL@PEG@anti-ER and AuNP@CV@PEG@anti-HER2 nanotags, b) PR^+HER2^+tissue using AuNP@MBA@PEG@anti-PR and AuNP@CV@PEG@anti-HER2 nanotags.*

Triplex analysis for tissue biomarkers

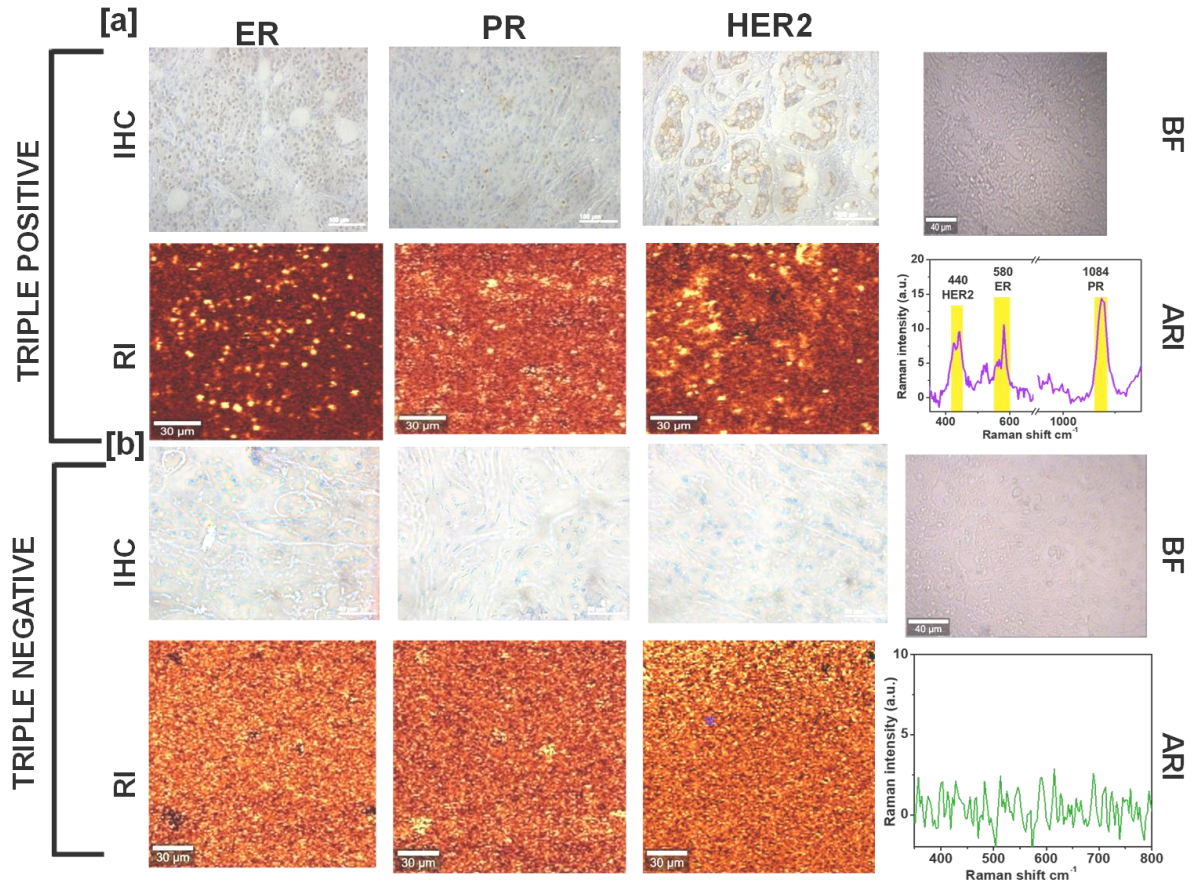
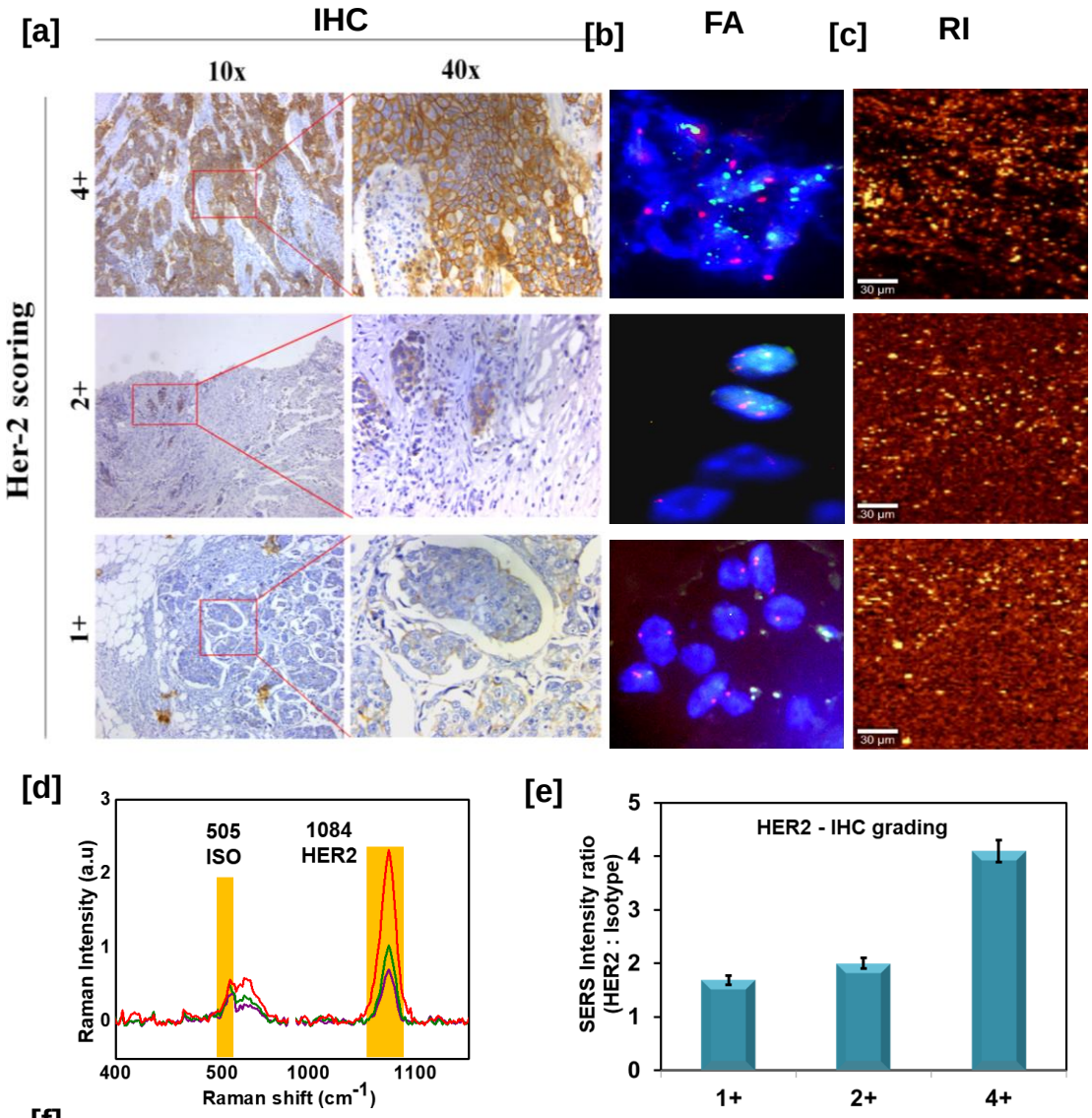


Figure 3: SERS analysis of a) $ER^{+}PR^{+}HER2^{+}$ and b) $ER^{-}PR^{-}HER2^{-}$ tissue using AuNP@SDL@PEG@anti-ER, AuNP@MBA@PEG@anti-PR and AuNP@CV@PEG@anti-HER2 nanotags. (BF-Bright field, RI-Raman imaging, ARI-Average Raman intensity, IHC-Immunohistochemistry)

HER-2 grading by SERS analysis.

Unlike ER and PR, HER2 over expression is considered for effective targeted therapy against the receptors like Herceptin treatment. IHC grading of 3+ and more are judged to be HER2 positive, whereas 2+ / equivocal expression has to be confirmed by FISH analysis in which the number of HER2 gene copies per nucleus is assessed. This method is again time consuming and highly expensive. Here we put forward a HER-2 grading system based on SERS intensity profile that can complement the IHC grading technique. For this, IHC grades of 1+, 2+ and 4+ HER2 tissue samples were incubated with AuNP@MBA@HER2 and AuNP@DTTC@Isotype antibody succeeded by SERS analysis as described earlier. DTTC was used as the Raman reporter for isotype antibody with 785 nm laser system and the same experiment was also executed in 633 nm laser with DTNB as the Raman reporter for isotype antibody. Ratio of HER2 tag to isotype tag obtained from the average scan intensities were plotted to get a mathematical interpretation of the same. Average SERS intensity from image scanning was offcourse higher for HER2 4+ tissue with an intensity ratio of 4.1 followed by HER2 2+ (Ratio

2) and 1+ (Ratio 1.79) in harmony with the IHC staining pattern, which was again confirmed by FISH analysis.



[f]

HER2 grade	A.R.I HER2	A.R.I ISO	HER2:ISO
1+	0.70	0.39	1.79
2+	1.01	0.52	2
4+	2.3	0.56	4.1

Figure 4: *SERS analysis showing HER2 grading in HER2+ tissue using HER2 targeted AuNP@CV@PEG@anti-HER2and Isotype targeted AuNP@DTTC@PEG@anti-isotype nanotags. a) IHC analysis, b) FISH analysis, c) Raman Imaging (RI), d) Average Raman Intensity (ARI), e)*

representation of HER2 grading by bar diagram and f) table showing ratiometric signal values of HER2 versus isotype tags.

Comparison of the technique of the present invention with IHC

Immunohistochemistry (IHC) is the existing gold standard method for detection of breast cancer biomarkers in formalin fixed paraffin embedded tissue samples. Table 2 provided below compares both the techniques in terms of specificity, easiness and time required for sample processing and analysis.

Table 1: Comparison of the features and main steps of conventional method IHC and SERS

Sl . No	Parameters	IHC	SERS
1	Multiplexing	Very difficult and absence of a standard method.	Easily Possible
2	Time required for sample preparation	7-27 hrs	4-6 hrs
3	Secondary antibody and developing agents	Required	Not required
4	Time required for analysis	0.5 hr/sample /one marker	1 hr/sample
5	Type of analysis	Highly Subjective(Inter observer variation)	Objective semi-quantitative
6	Grading	Based on percentage of stained cells and stain intensity	Based on spectral intensity based mapping
7	HER2 grading	2+/Equivocal samples required FISH confirmation	By ratiometric semi quantitative approach FISH confirmation may be mitigated

Major Advantages and Conclusion

A rapid multiplexed detection of clinically relevant biomarkers in FFPE tissue is an essential criterion for enabling efficient treatment strategies in heterogeneous breast cancer.

The study thus reveals a new diagnostic modality with huge potential for the detection of tumors as well as tumor recurrence exhibiting differential biomarkers associated with patient-to-patient heterogeneity.

Key Advantages

1. In the present invention, SERS analysis i.e., scanning, and imaging of the SERS-nanotag is performed to gather the information from maximum locations in order to know the abundance of biomarkers in the breast tissue sample.
2. In this modality, a SERS intensity based semi-quantitative system for HER-2 gradation has been provided using the SERS-nanotag since the over expression of HER-2 (2+ and above from immunohistochemistry grading) is considered by the clinicians to judge the samples as positive.
3. Simultaneous recognition of breast cancer biomarkers ER, PR, HER2, and Ki67 expression in a single detection mode with a single laser utilizing respective antibody conjugated SERS-nanotag of the present invention is termed as SERS based immunoassays.
4. Simultaneous detection modality is achieved by initial validation in paraffin embedded breast cancer tissue samples. By evaluation of SERS signature peaks from the respective nanotags, ER, PR, HER2 and Ki67 status from the tissue sample is confirmed which definitely propagates into treatment management with high precision, minimum assay time, and in a cost-effective manner.
5. The nanotag of the present invention is highly accurate and there is very low possibility of false positive and false negative results.

References:

Cervical Cancer detection:

Patent:

Screening kit for detection of grades of cervical cancer and process for the preparation thereof; Maiti, Kaustabh Kumar, Varsha Karunakaran, K.Sujathan; ***PCT Int. Appl. (2020), WO 2020021568 A1 20200130. Language: English, Database: CAPLUS, Date: 30th January, 2020***

Publication:

Diagnostic Spectro-cytology revealing differential recognition of cervical Cancer lesions by label-free surface enhanced Raman fingerprints and Chemomssetrics; Varsha Karunakaran, Valliamma N. Saritha, Manu M.Joseph, Jyothi B. Nair, Giridharan Saranya, Kozhiparambil G. Raghu, Kunjuraman Sujathan*, Krishnan Nair S. Kumar*, Kaustabh K. Maiti* **Nanomedicine: Nanotechnology, Biology and Medicine**, 2020, 29, 102276

Multiplex Breast Cancer Biomarker Detection

Patent:

A SERS-Nanotag and a Diagnostic kit for the Detection of Breast Cancer Biomarkers; Maiti, Kaustabh Kumar, K. Sujathan, Vishnu Priya Murali, Varsha K, Deepika S, Madhukrishnan M ; ***Indian Patent Application ref. No. 202011034768, dated 11.08.2020***

PCT Application No. PCT/IN2021/050577, dated 14.06.2021

Kaustabh Kumar Maiti 22/09/2021

डॉ. कौस्ताभ कुमार मैती / Dr. KAUSTABH KUMAR MAITI
प्रधान वैज्ञानिक / Principal Scientist
रसायन विज्ञान तथा प्रौद्योगिकी प्रभाग
Chemical Sciences and Technology Division
सी एस आई आर- राष्ट्रीय अंतर्विषयी विज्ञान तथा प्रौद्योगिकी संस्थान
CSIR-National Institute for Interdisciplinary Science and Technology (NIiST), Govt. of India
तिरुवनन्तपुरम/Thiruvananthapuram-695 019