Imaging Mass Spectrometry Analysis Enables *In-Situ* Detection of Human Papillomavirus in FFPE Tissues

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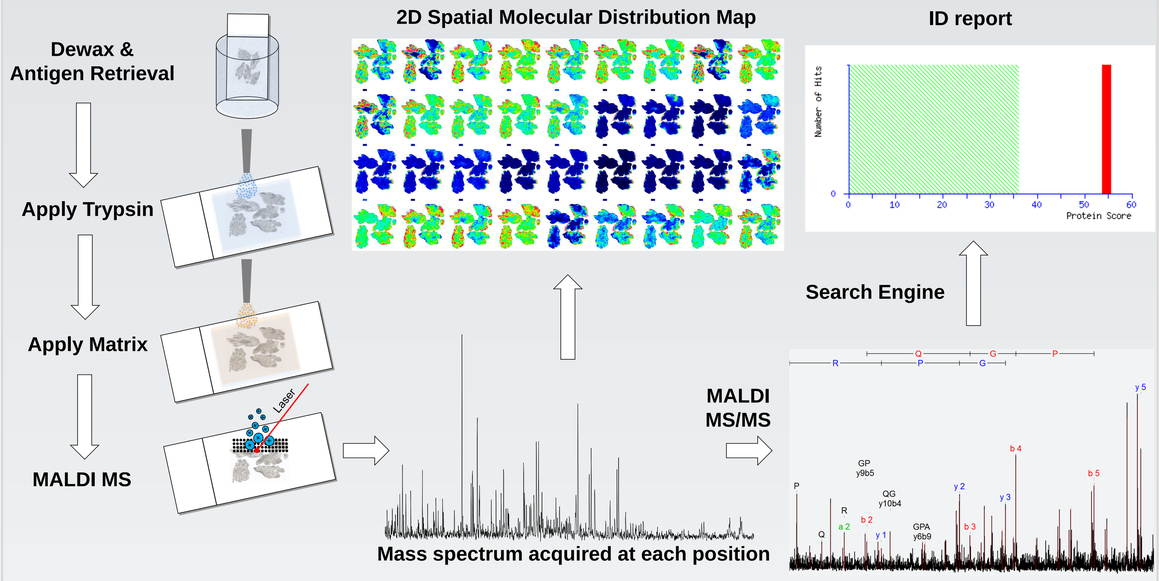
Objective: Imaging mass spectrometry (IMS) application for the detection of human papilloma virus (HPV)-related proteins directly in the host specimen.

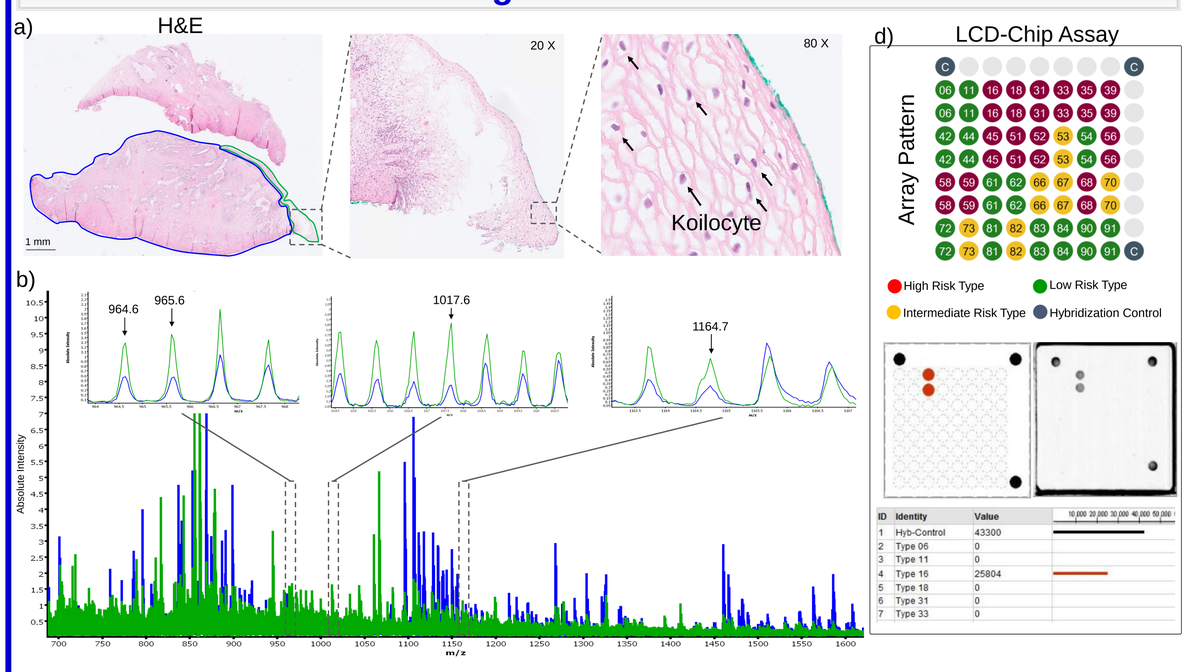
Introduction: HPV comprises a family of more than 130 types, with many types having preference to infect specific anatomical sites, causing lesions with distinctive clinical pathology. These include benign hyper-proliferative lesions such as cutaneous warts, and asymptomatic precursor lesions, that can in some instances progress to high-grade neoplasia and invasive cancer. Test systems are required to detect high-risk but also low-risk HPV subtypes with high specificity and sensitivity. We developed a proteomic-based IMS approach to investigate HPV molecular signals directly from host specimens.

Methods and Workflow: Formalin-fixed paraffin-embedded tissue sections from cervical cancer, genital and skin warts were prepared for IMS analysis according to an optimized standard protocol[1]. Briefly, tissues were deparaffinized with xylene, rehydrated through graded ethanol and antigen retrieved in 10mM Tris buffer (pH 9) for 20 min at 95°C. Sections were sprayed with trypsin (0.025 µg/µL) using a TM-Sprayer (HTX Technologies, Chapel Hill, NC, USA) and incubated at 37°C for 2 hours. Alpha-cyano-4-hydroxycinnamic acid matrix solution (10mg/ml in 70%ACN/1% TFA) was deposited using the same sprayer device.



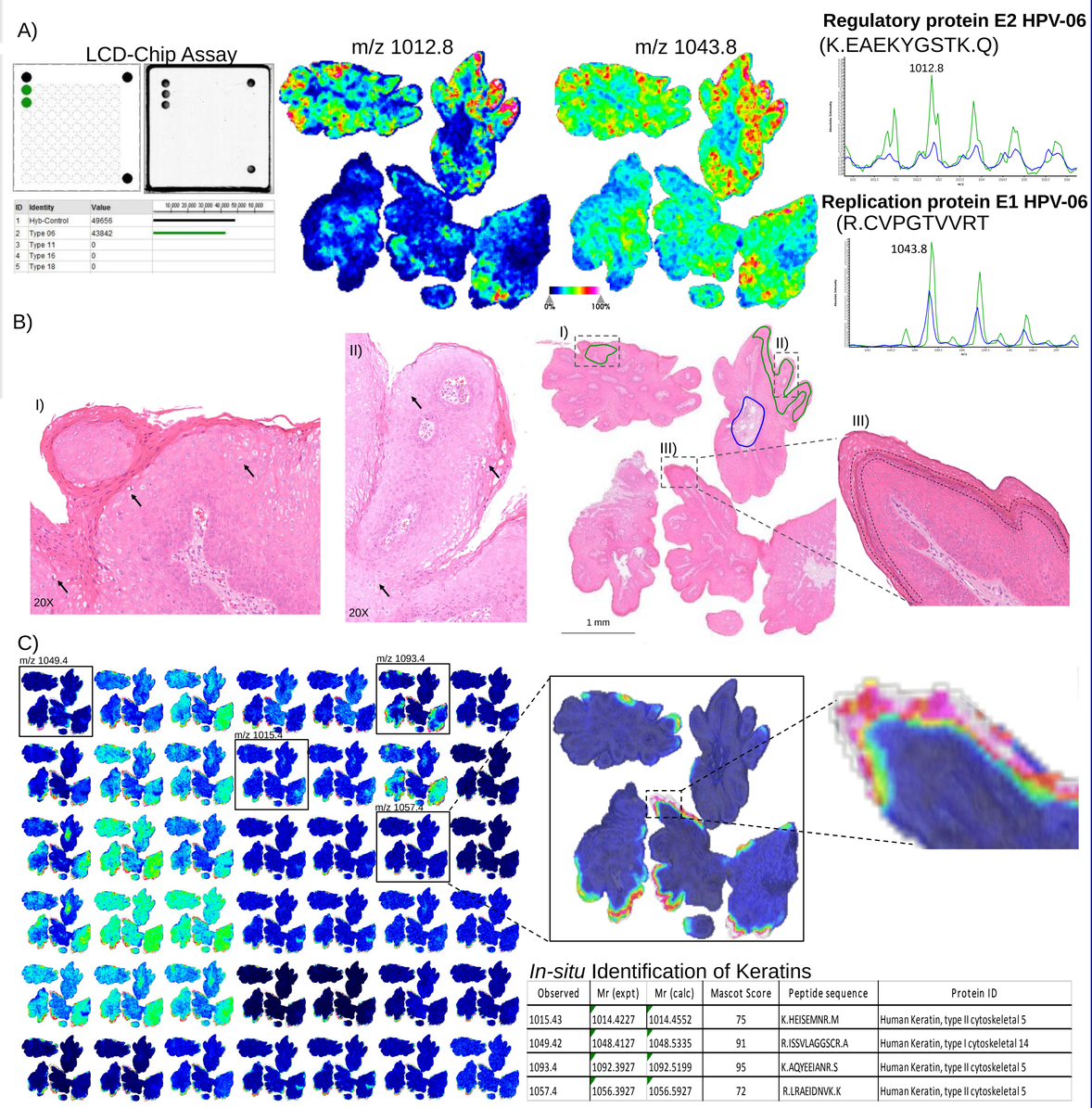
MS measurements for IMS were carried out using a rapifleX MALDI Tissuetyper (Bruker Daltonik GmbH, Bremen, Germany) at 50µm spatial resolution. Spectra were exported from HPV infected and non-infected regions and compared. SCiLS Lab software (SCiLS, Bremen, Germany) was used to automatically find m/z values specifically co-localizing within the HPV infected annotated regions using a statistical Pearson correlation method with p≤0.05. Protein identification was performed *in-situ* using an AutoflexSpeed MALDI-TOF/TOF (Bruker Daltonik) mass spectrometer.

IMS Detection of High-Risk HPV in Cervical Cancer



Mass spectra comparison between HPV infected (outlined in green on the HE stain) and non-infected (outlined in blue on the HE stain) regions showed differential peptide expression (b). Four peptides were specifically distributed in the HPV-infected region which show features typical of koilocyte cell. These ions were *in-situ* identified as regulatory protein E2 (m/z 964.6), major capsid protein L1 (965.6, 1017.6) and protein E4 (1164.7) of HPV type 16 (c). This result was in agreement with the routine hybridization-based test system HPV LCD-array kit (Chipron) (d).

IMS Analysis of Anogenital Wart Infected with Low-Risk HPV

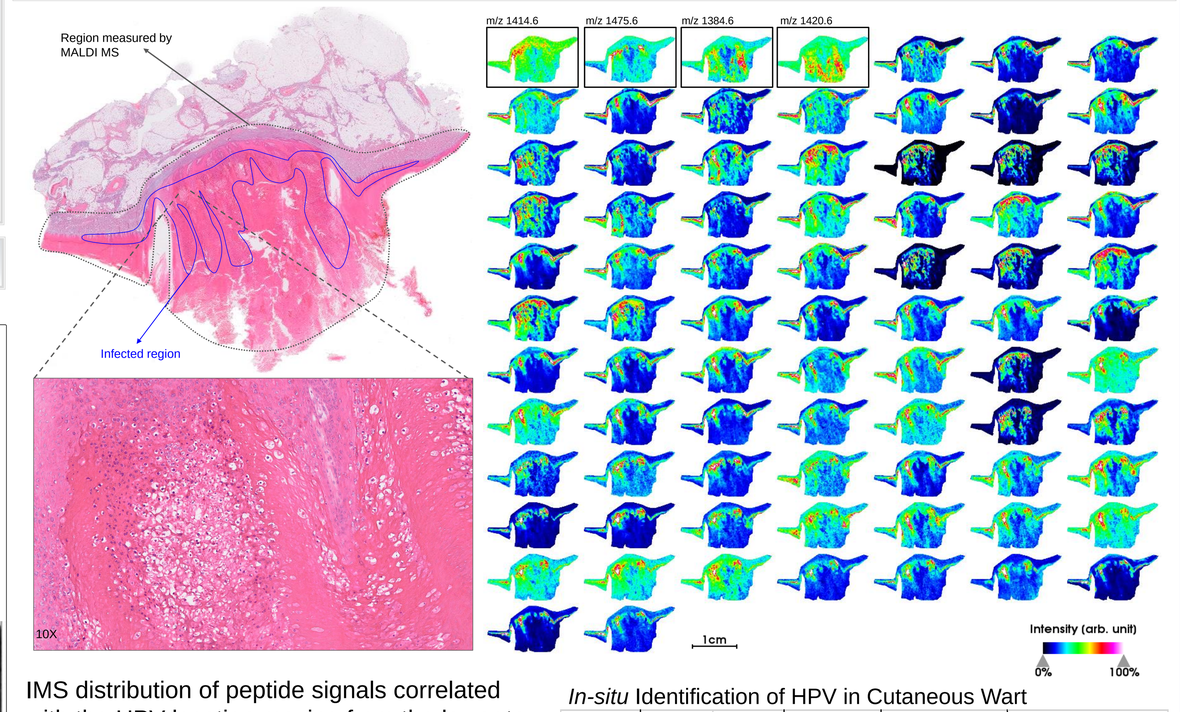


In-situ Identification of Keratins

Observed	Mr (expt)	Mr (calc)	Mascot Score	Peptide sequence	Protein ID
1015.43	1014.4227	1014.4552	75	K.HEISEMNR.M	Human Keratin, type II cytoskeletal 5
1049.42	1048.4127	1048.5335	91	R.ISSVLAGGSCR.A	Human Keratin, type I cytoskeletal 14
1093.4	1092.3927	1092.5199	95	K.AQYEEIANR.S	Human Keratin, type II cytoskeletal 5
1057.4	1056.3927	1056.5927	72	R.LRAEIDNVK.K	Human Keratin, type II cytoskeletal 5

A) MS images and profiles of two ion peptides identified as regulatory protein E2 (m/z 1012.8) and replication protein E1 (m/z 1043.8) of HPV-06 type, correlating HPV infected regions of an anogenital wart diagnosized with low risk HPV type 06 by LCD-Chip Assay test. Average spectrum in green is related to infected regions (green line on the HE stain, B-II), average spectrum in blue represents a non-infected region (blue line on the HE stain, B-II). B) HE stain tissue after MALDI analysis showing infected area (green line) by the presence of koilocytes indicated with black arrows (I, II). C) MS images of peptides correlating keratinocytes (dashed line on the HE stain, B-III). Cytokeratin 14 (m/z 1049.4) and cytokeratin 5 (m/z 1015.4, 1093.4, 1057.4) human proteins were identified with high intensity colocalizing with high proliferative activity in keratinocyte cells (tissue images outlined in black squares).

Identification of HPV in Cutaneous Wart



IMS distribution of peptide signals correlated with the HPV location moving from the lower to the top epidermis skin layers of a cutaneous plantar wart that has not been subjected to routine diagnostic HPV test. Four proteins were identified to be related to three HPV types, specifically, replication protein E1 and major capsid protein L1 of HPV 2 type (m/z 1414.6, 1475.6), replication protein E1 of HPV type 27 (m/z 1384.6), and major capsid protein L1 of HPV 57 (m/z 1420.6). Peptide IMS distribution of the identified proteins are outlined in black squares.

In-situ Identification of HPV in Cutaneous Wart

Observed	Mr (expt)	Mr (calc)	Mascot Score	Peptide sequence	Protein ID
1384.6	1383.5927	13863.6493	117	K.NCIVFYGPADTGK.S	Replication protein E1-HPV 27
1414.6	1413.5927	1413.7293	83	K.DLFGVGFYELVR.Q	Replication protein E1-HPV 2a
1420.6	1419.6327	1419.6738	82	K.SDVPLDICTNICK.Y	Major capsid protein L1 HPV 57
1475.6	1474.6627	1474.8078	77	K.FLLQRGAMPTVSR.K	Major capsid protein L1 HPV 2a

Conclusions: Specific peptides related to individual HPV types were imaged and identified directly onto host specimens using IMS. Our results were in agreement with hybridization-based test systems used in routine diagnostics. Co-infections of a single wart with multiple HPV types were detected. The ability to rapidly identify signatures specific to microorganisms in tissue is a major advantage that greatly decrease both time and cost especially for the analysis of tissue with multiple simultaneous HPV infections.

References: [1] Ly A, Longuespée R, Casadonte R, Wandernoth P, Schwamborn K, *et al.* Site-to-Site Reproducibility and Spatial Resolution in MALDI-MSI of Peptides from Formalin-Fixed Paraffin-Embedded Samples. Proteomics Clin Appl. 2019 Jan;13(1).

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