


Ram**A**n – not as delicious, but at least as versatile



Raman Spectroscopy for label-free cell type identification

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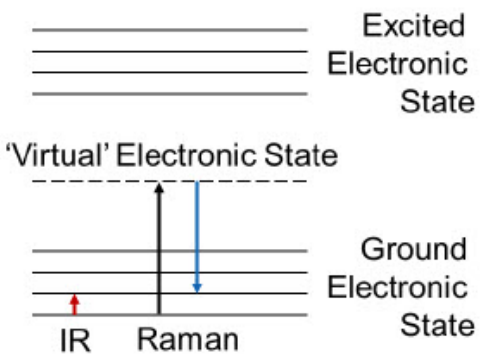
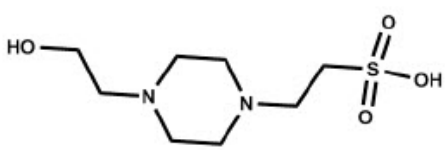
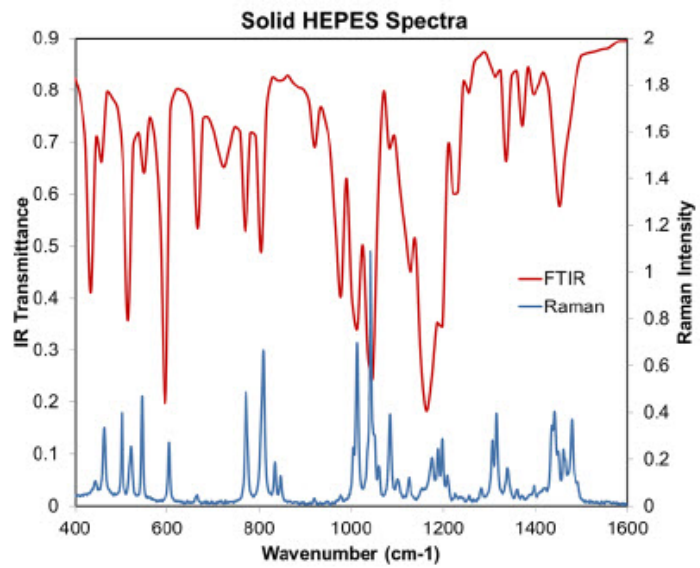
What is Raman scatter?

- Raman = indirect measure of the vibrational spectrum (FTIR = direct vibrational absorbance)
- Raman spectroscopy exposes the sample to monochromatic light → some of the scattered light is shifted to a lower energy, since some molecules in the sample got a higher vibrational state.
- Raman scattering increases with light energy (but more background)







Sigmaaldrich.com

How is the data collected and analyzed?

Components:

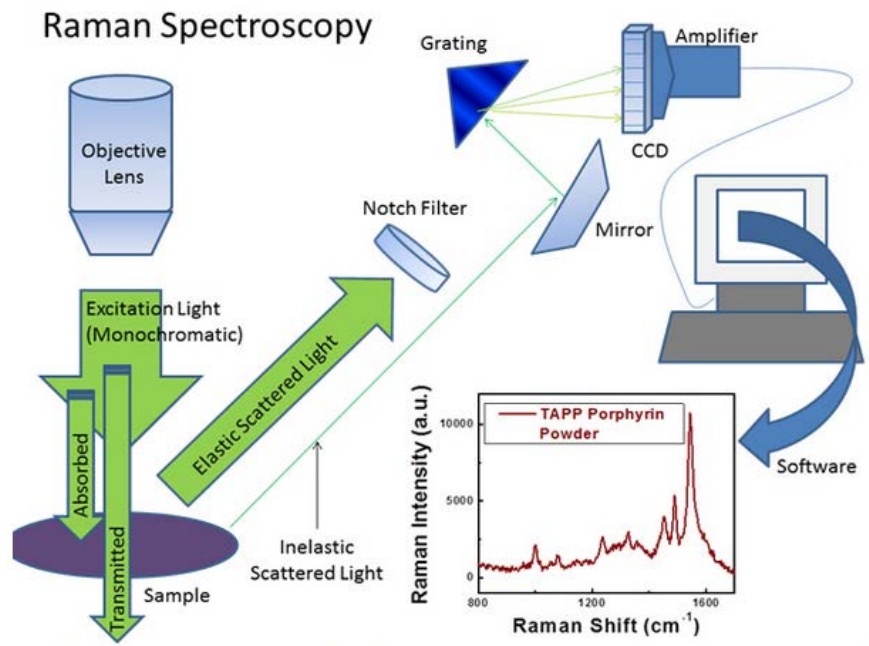
- laser
- sampling interface
- spectrometer

Laser characteristics:

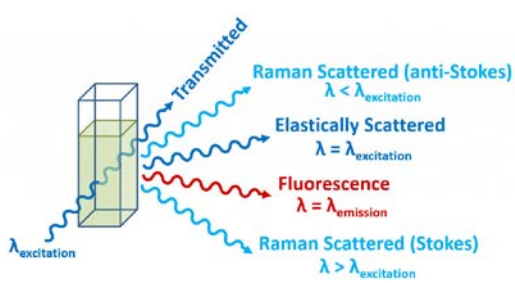
- small form factor
- low power consumption
- narrow linewidth
- stable power output
- stable wavelength output

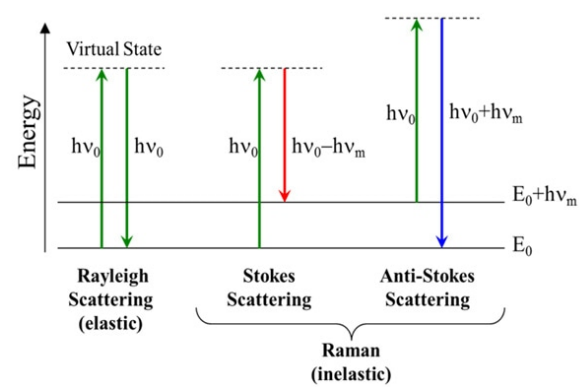
Setup characteristics:

- TE cooled spectrometer to collect weak signal with reduced noise
- Thinned CCD camera for higher sensitivity
- High resolution spectrometer to resolve closely spaced peaks (standard lasers: 785nm, 532nm)



source: https://www3.nd.edu/~kamatlab

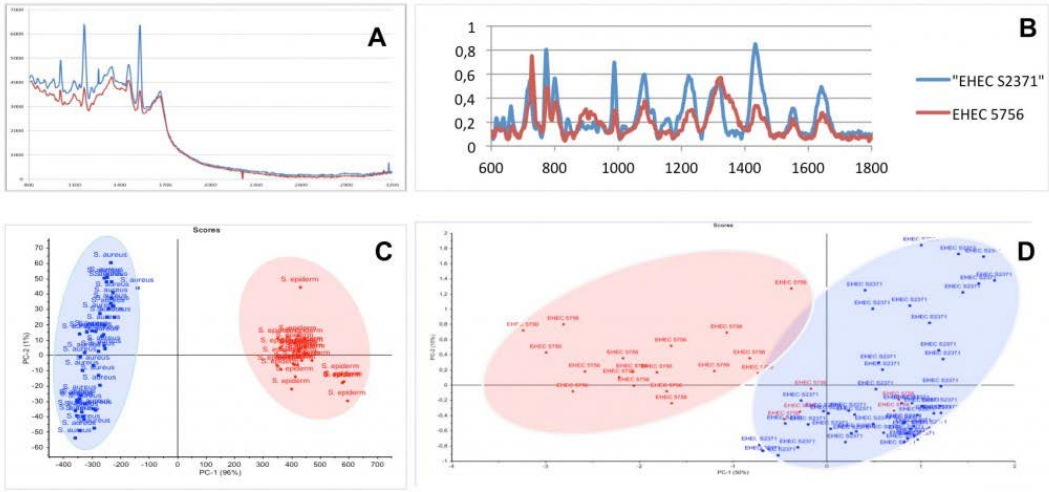




How is the data collected and analyzed?

Data analysis:

- preprocessing stage: reducing the effect of unwanted signals
  - cell media,
  - glass substrate,
  - fluorescence,
  - detector noise,
  - calibration errors,
  - cosmic rays,
  - laser power fluctuations



- Multivariate data analysis
  - Unsupervised methods
  - Supervised methods

- Classification models
  - Linear Discriminant Analysis (LDA)
  - Soft Independent Modeling of Class Analogy (SIMCA)
  - Artificial Neural Networks (ANN)
  - Support Vector Machines (SVM)

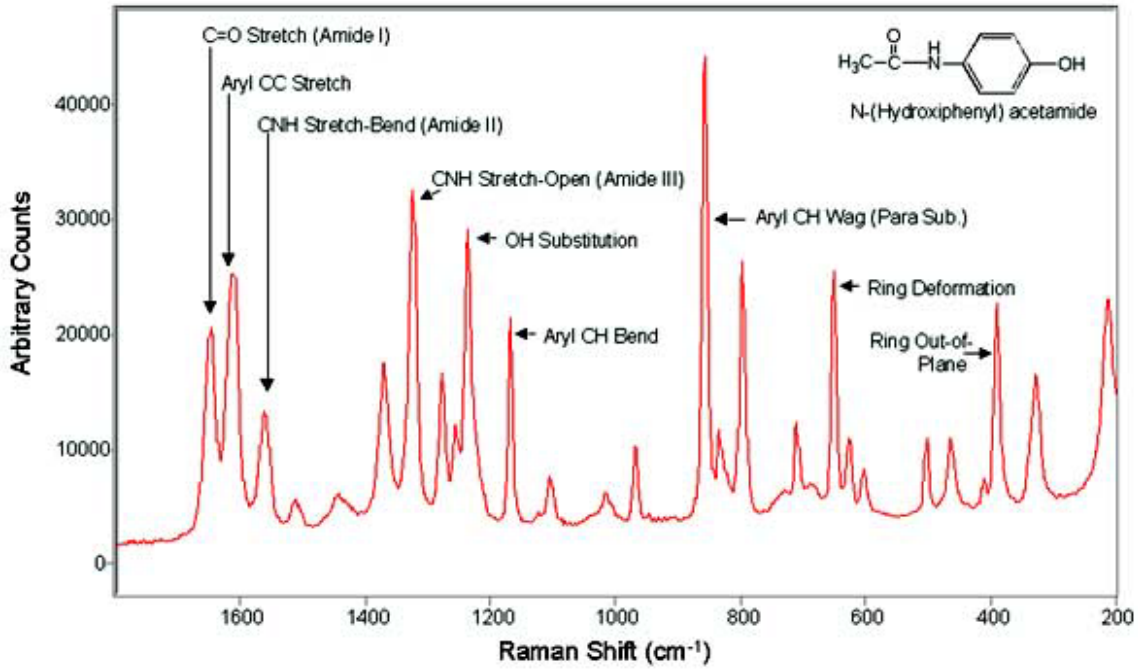
- Statistics

http://www.int.laborundmore.com/archive/358356/Raman-spectroscopy-for-biologists-and-clinicians.html

How to interpret a Raman spectrum?

- Photons are forced to leave their straight path by interaction with sample
- Laser light interacts with molecular vibration causing up/downshift of laser energy
- Bonds in the sample can be identified by analyzing the energy shift
- Spectrum is unique to a material → good for identification of compounds

Functional group	Region
C−C	~600-1300 cm^{-1}
C=C	~1600 cm^{-1}
C≡C	~2100-2300 cm^{-1}
C−H	~2700-3100 cm^{-1}



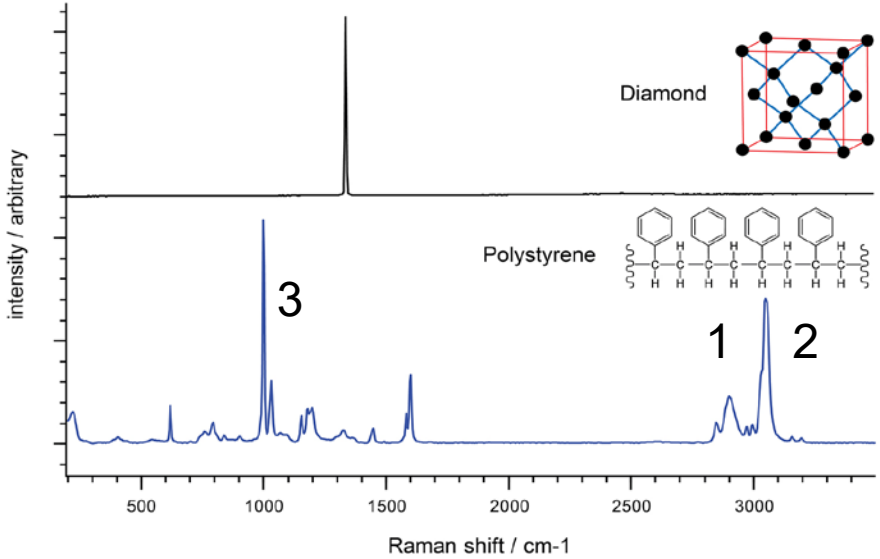
How to interpret a Raman spectrum?

- Polystyrene: less symmetric molecule with hydrogen and carbon & different bond types – vibration frequencies depend on atom mass and bond strength:
  - heavy atoms, weak bonds = low Raman shift
  - Light atoms, strong bonds = high Raman shift

C-H vibration ~3000 cm-1 and C-C ~800 cm-1, C=C ~1600 cm-1,

- strength of bonds affects vibration
  - C-H vibrations 2900 cm^{-1}: aliphatic carbon chains (1)
  - C-H vibrations 3050 cm^{-1}: aromatic carbon rings (2)
  - Vibrations at 1000 cm^{-1}: whole aromatic carbon ring vibration (3)

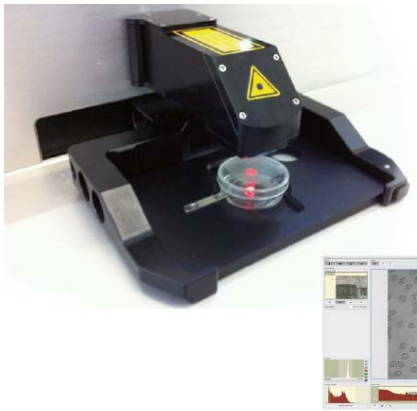
- Diamond: crystals with regular identical atoms in same configurations



How can Raman spectroscopy be used?

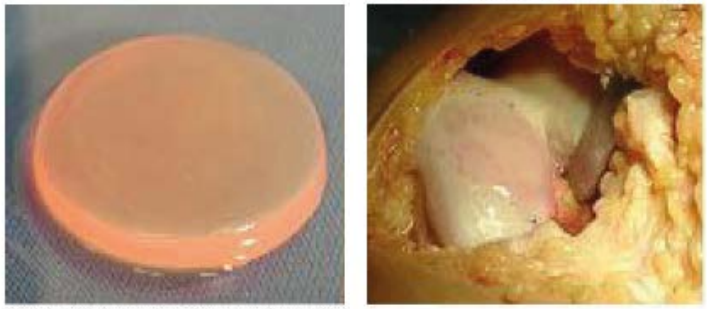
Fields:

- material science
- food industry
- medicine
- analytical chemistry
- Forensics etc.



Analysis of:

- different cell states (e.g. cancer reserach)
- rare cell analysis
- interactions (cell-compound)
- toxicology
- questioned documents
- biological samples
- trace analysis
- explosives and GSRs etc.



Graft from autologous cartilage biopsy materia

Advantages for life science:

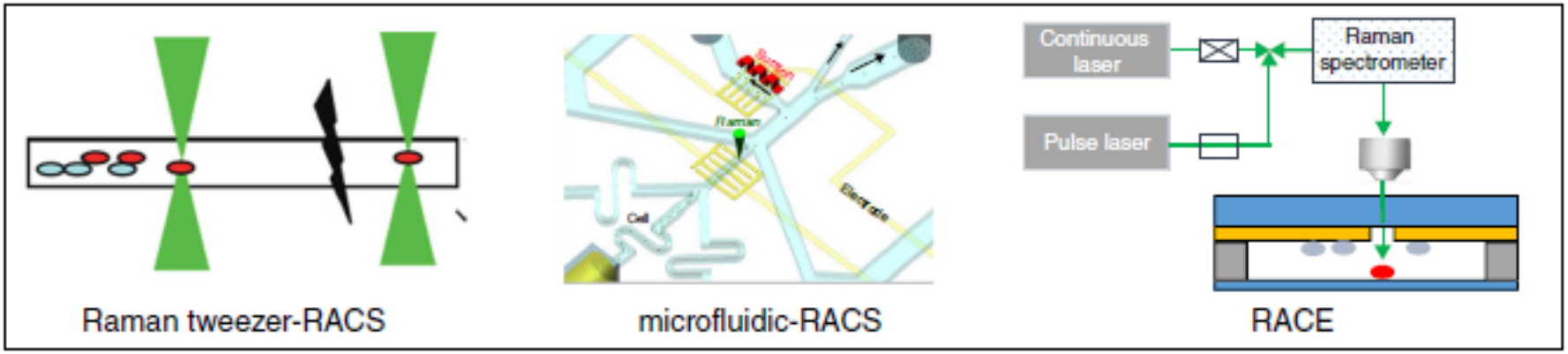
- Stable/good cell viability
- no changes applied e.g. cells for therapies can be sorted without alterations
- Cultured tissue can be analyzed without disruption
- Small sample volume
- rare cells identification
- 785nm laser for biological samples → no cell damage, minimally absorbed by aqueous solutions

Raman based cell sorting

Raman-activated cell sorting (**RACS**):

- No need for external labelling
- Consists of single cell Raman spectra analyzer (SCRS) & a cell isolation system
  - in solution (Raman tweezers)
  - in flow (microfluidic based RACS)
  - on a surface (Raman activated cell ejection — RACE)

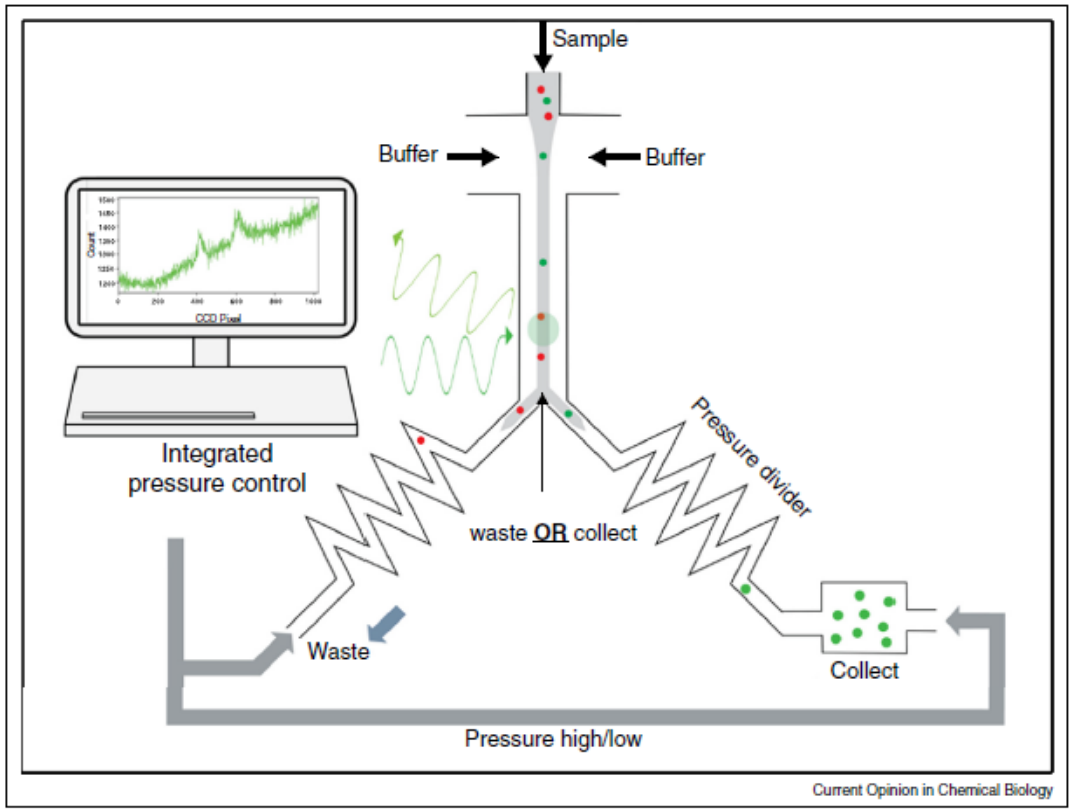
RACS in solution: Optical tweezers - highly focused laser beam gives attractive or repulsive force to hold and move neutral objects



Raman based cell sorting

trap-free RACS in flow (microfluidic based):

- simple hydrodynamic focusing
- pressure switch mechanism to sort individual cells based on Raman signals



Raman based cell sorting

On a surface: Raman activated cell ejection (RACE)

- to maintain spatial organization of cells & to understand interactions and functions
- RACE can isolate cells of interest in their biological niches
- 337 nm pulsed laser used to isolate single cells by laser induced forward transfer (LIFT)
- Cells are mounted on specific slide coated with laser absorbing material & can be ejected using LIFT

Yizhi Song, Current Opinion in Chemical Biology 2016, 33:1–8

Fluorescence vs. Raman activated cell sorting

FACS:

- External labelling – requires previous knowledge of the sample
- Information restricted (Abs, lasers, physical parameters)
- Cells in suspension
- Fast (up to 5000 cells/s)

Raman tweezer RACS:

- External label free (maybe intrinsic stable isotope labelling) – no pre-knowledge needed
- Information about biochemical and metabolic features (lipids, proteins, carbohydrates, nucleic acids..)
- Cells in suspension
- Slow (~3min/cell)

Microfluidic RACS:

- Slow (5-100 cells/s)

RACE:

- Cells in suspension, tissue, attached to solid surface
- 1cell/s

(Coherent anti-Stokes Raman spectroscopy (CARS) and stimulated Raman scattering (SRS) overcome naturally weak signal of spontaneous Raman scattering and significantly shortening Raman acquisition time)

Raman spectroscopy for cell identification

Meso-Raman approach for rapid yeast cells identification

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Raman micro-spectroscopy for accurate identification of primary human bronchial epithelial cells

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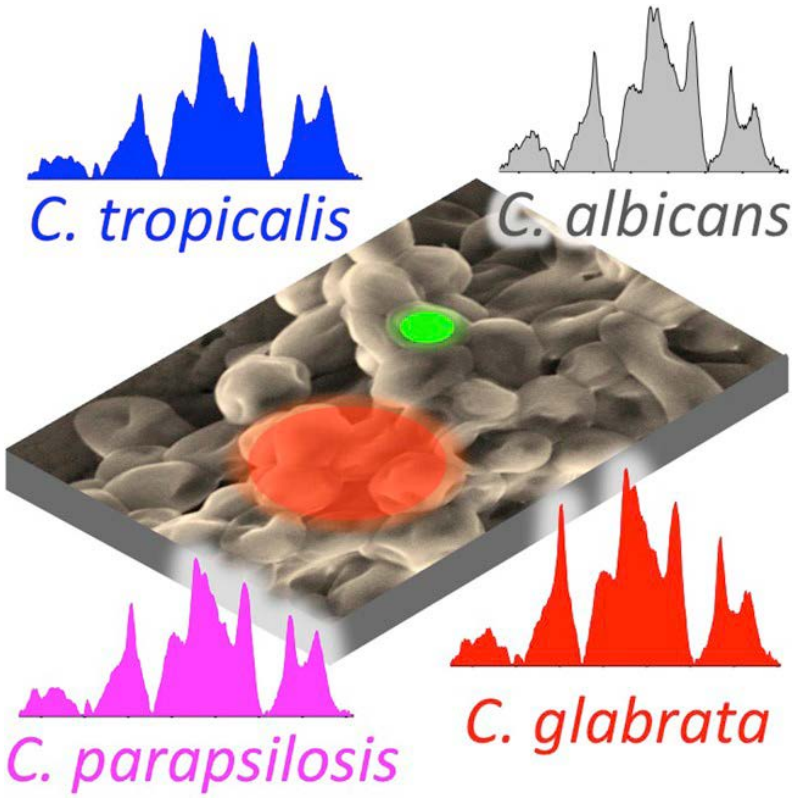
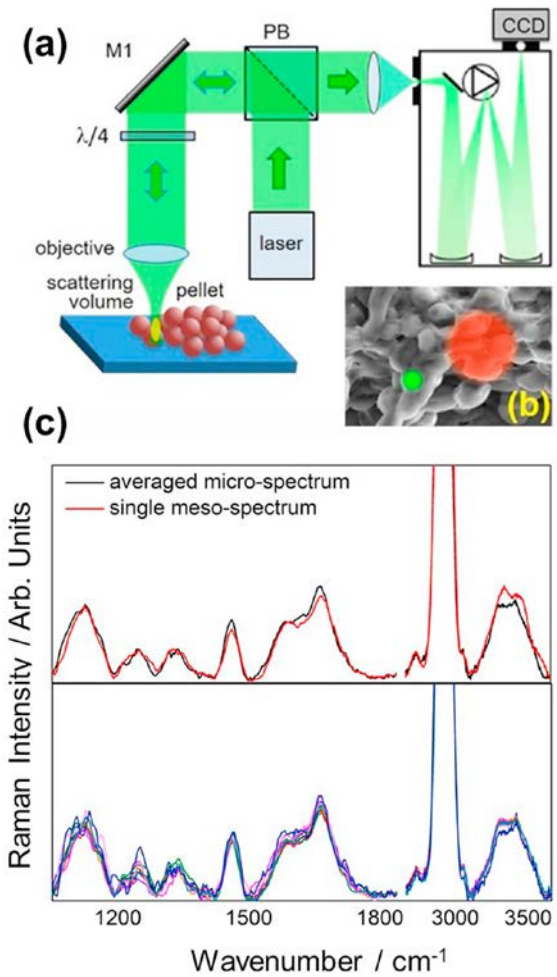
Meso-Raman approach for rapid yeast cells identification

- A meso-Raman approach for yeast cell identification
- Unpolarized and Depolarized Raman Spectra for PCA analysis
- Future efficient and reliable protocol for medical diagnosis in clinics



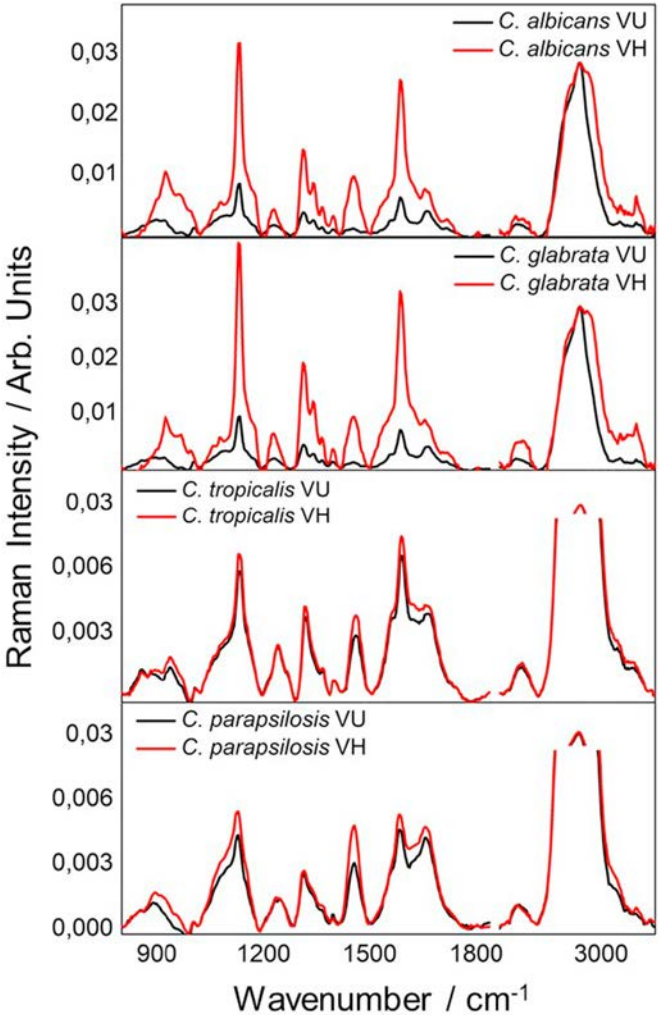
Setup and comparison of micro- and meso Raman spectra

- Schematic of the Raman spectroscopy setup
  - 532nm laser
  - Spectra in unpolarized (VU) and depolarized (VH) configurations
- SEM micrograph of the *C.albicans* film
  - Green dot: 2 μm splot of the 20mm lens
  - Red dot: 8 μm spot of the 30mm lens
- Lower panel: ten spectra a yeast cells film using the 20mm objective lens
- Higher panel: comparison between average of ten spectra and single spectrum collected by the 30mm lens



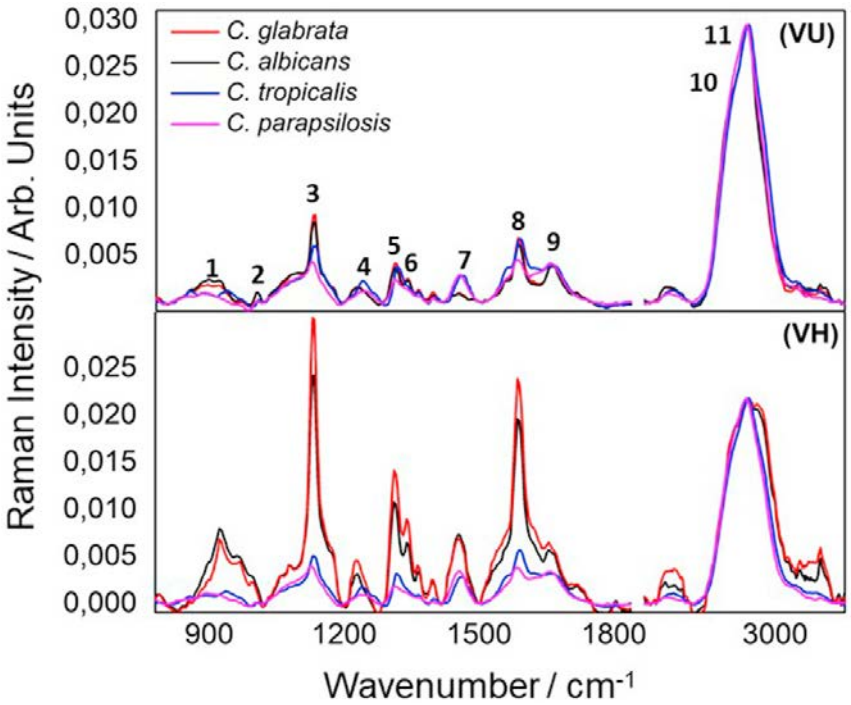
Different symmetry of vibrational modes induce differnt peak intensities in unpolarized vs. depolarized mode

- Raman averaged spectra of *Candida* films after fluorescent background subtraction and normalization to high frequency
- CH2-CH3 stretching bands recorded using the depolarized and unpolarized configurations
- Different symmetry of vibrational modes of biomolecules (caused by different interactions with local environment) differ in de- and unpolarized configuration



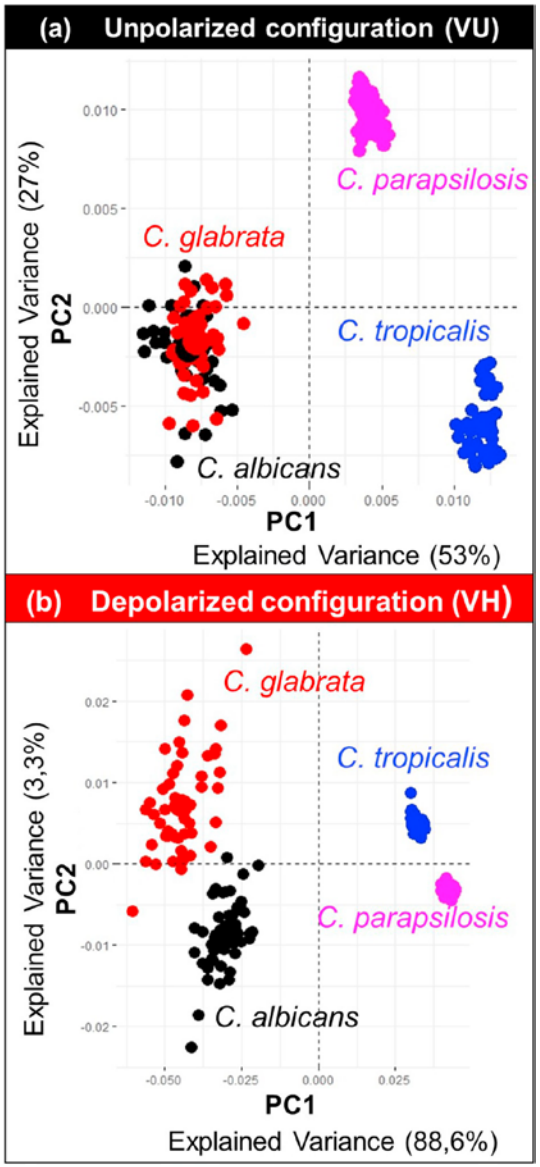
Comparison of averaged Raman spectra in depolarized and unpolarized configuration

- Average of 50 spectra in de-and unpolarized mode
- 800 - 3100cm^{-1} investigated
  - Except “silent region” 1780-2640 cm^{-1}
- depolarized (upper panel)
- unpolarized (lower panel)



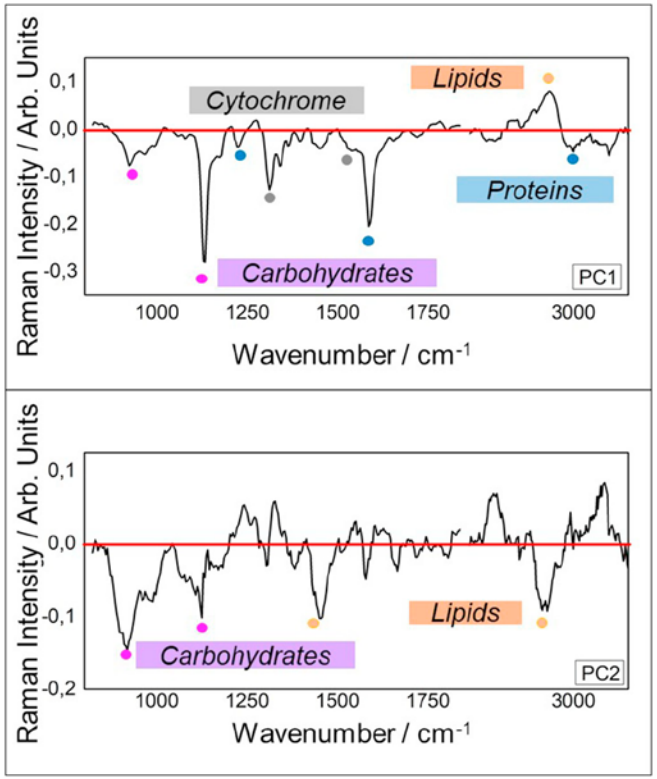
PCA analysis on VU & VH spectra shows value of VH configuration

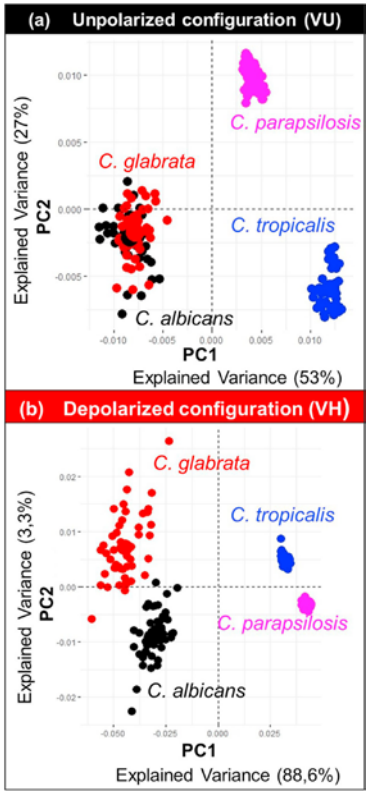
- PCA plot obtained from VU spectra and from VH spectra of four different pellets:
  - ***C. tropicalis***
  - ***C. parapsilosis***
  - ***C. albicans***
  - ***C. glabrata***
- Clear discrimination of only 3 groups using VU spectra
- Using VH spectra, samples can be divided into 4 groups
  - Baseline substraction may play a major role in distinguishing C. albicans and C. glabrata
- Spectroscopic data in different polarization helps identifying these species
- Candida samples are highly luminescent - basleine substraction is important



Shape analysis of PC1 & PC2 gives further information

- Projection of the loadings plot on the PC1 &PC2
- PC1 can distinguish **C. tropicalis** and **C. parapsilosis** form **C. albicans** and **C. glabrata** due to different relative protein and lipid content (high frequency CH stretching band profile)
- PC2 distinguishes **C. glabrata** and **C. tropicalis** from **C. albicans** and **C. parapsilosis**. Frequences assignable to carbohydrates (glucose, glycogen) and frequencies characteristic of lipid components mostly present on **C. albicans** and **C. tropicalis** spectra.





Conclusions & Significance

- Established a possible procedure for Raman phenotypical identification of yeast cells using meso-Raman approach
  - Meso-Raman records averages of tens of cells → reduced acquisition time and facilitated data processing
  - Reduced spectral variability (metabolic states, colony architecture, cellular portions)
  - Most common Candida species could be distinguished using PCA analysis including depolarized spectra
  - Large scale microbial identification possible, since no selection of cell portion for analysis is needed
- Low price, low biomass needed (short incubation times)

Raman spectroscopy for cell identification

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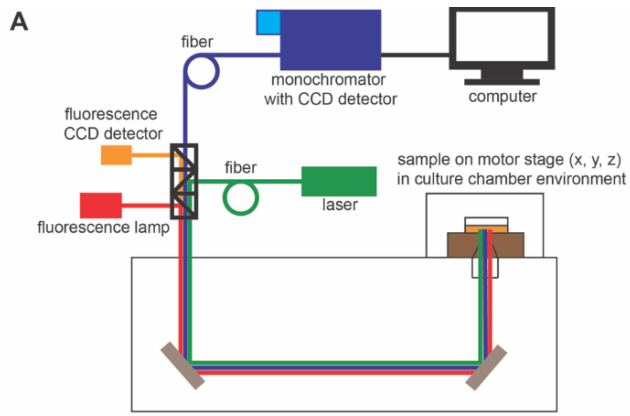
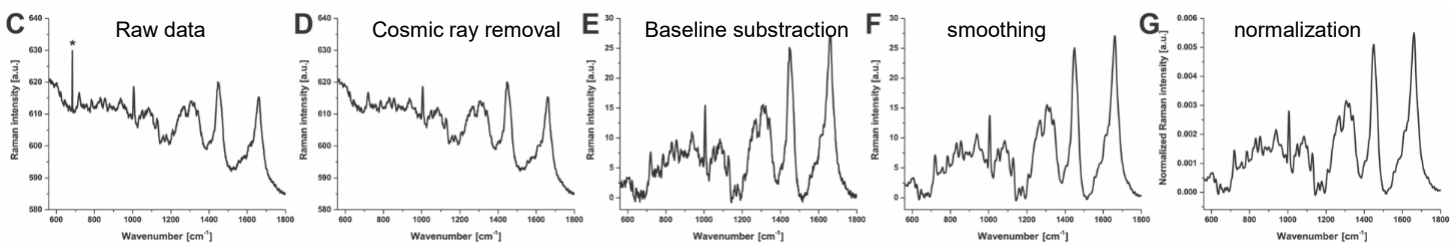
Raman micro-spectroscopy for accurate identification of primary human bronchial epithelial cells

- Development and application of optimized protocol for confocal Raman micro-spectroscopy to identify different living human lung cell lines in culture

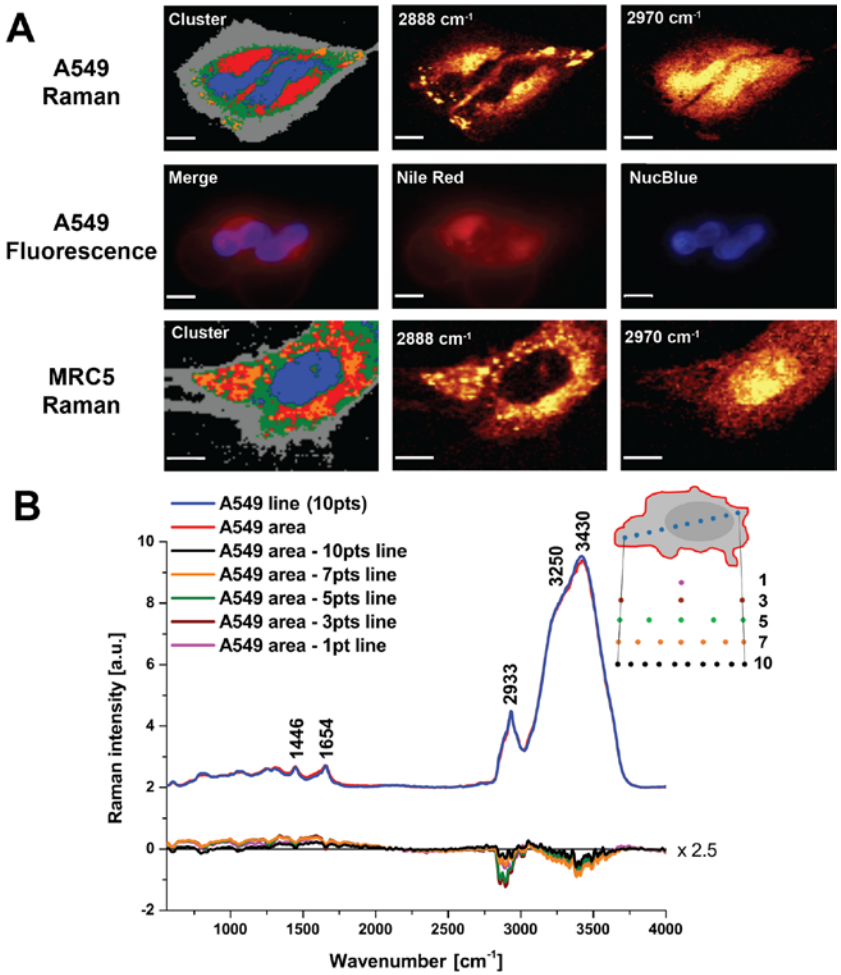
- Delineation of immortalized human cell lines derived from lung cancer (A549), fibroblasts (MRC5) and 3 primary human bronchial epithelial cell (HBEC) lines based on lipid composition, lipid droplets and differences in DNA content





Comparison of line scan data acquisition from A549 and MRC5 cells

- Comparison line scan vs. Area scan to increase the analysis speed
- Comparison of Raman vs. Fluorescence
  - MRC5 cells could not be compared to fluorescence: migratory cells
- Area scans using 488 & 785 nm, combined with quartz substrate used
- Area scan (488nm) with image production using k-means clustering and sum filters to optimize the procedure
- Fluorescence images to compare lipid rich regions & nuclei
  - NucBlue for nucleus
  - NileRed for lipids
- Averaging spectra from line spanning a single cell – using 10 points could recapitulate the area spectrum in short time



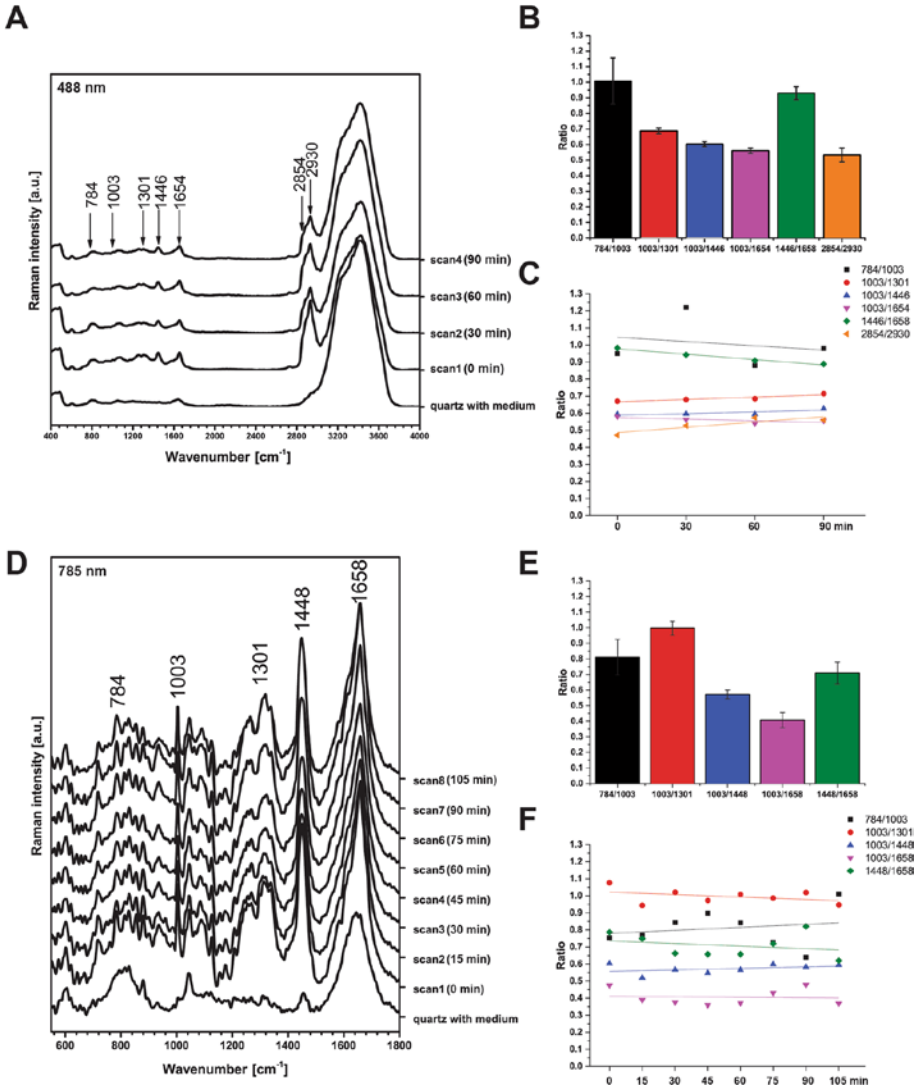
Impact of longitudinal line-scan data acquisition at multiple time points on live cell spectra

Testing the impact of line scan acquisition on the cell`s biochemistry

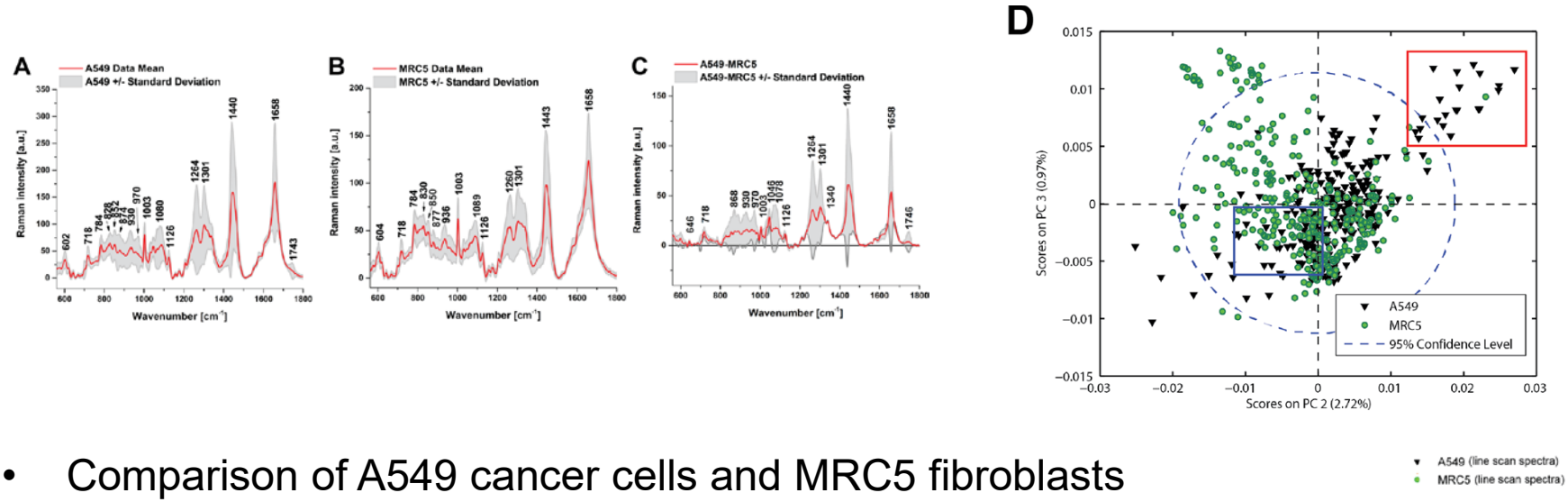
- Repeated scan of same cell over 2 hours with 488 & 785 nm

- No significant change of peak intensity ratios, but trend to be altered after 2 hours using 488 nm

- For 785 nm intensity ratios seem unaltered after 2 hours repeated measurment → preferred illumination for repeatedly analyzing the same cell



Analysis of live human immortalized lung cell lines

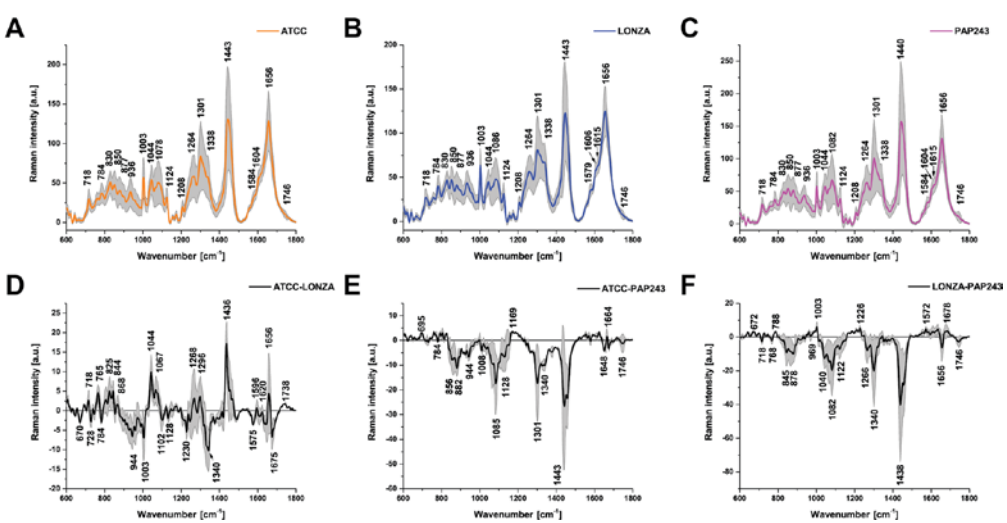


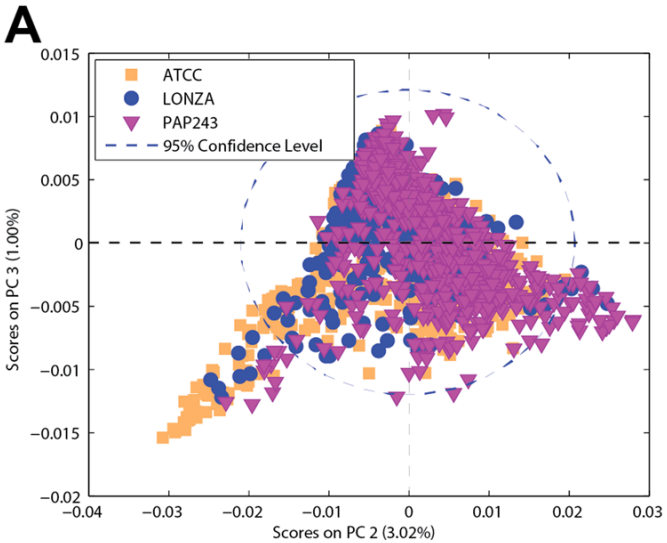
- Comparison of A549 cancer cells and MRC5 fibroblasts
  - 10 point line scan experiments over 30 cells each
- Variability within lines is sufficiently low to detect differences between lines (DNA band, lipids and proteins)
- PCA analysis reveals presence of lipid droplets as distinguishing factor (PC3 higher in A549 cells)

Identification of primary human bronchial epithelial cells in culture

- 10 point line scan experiments over 30 cells for 3 different primary HBEC lines
  - in house, patient derived (never moker, carcinoid diagnosed)
  - Smoker, non-cancer (ATCC)
  - Never-smoker, non-cancer (LONZA)
- Assessing the ability to distinguish the culutures by comparing mean spectra, difference spectra and PCA (key differences from DNA, Lipids and proteins)

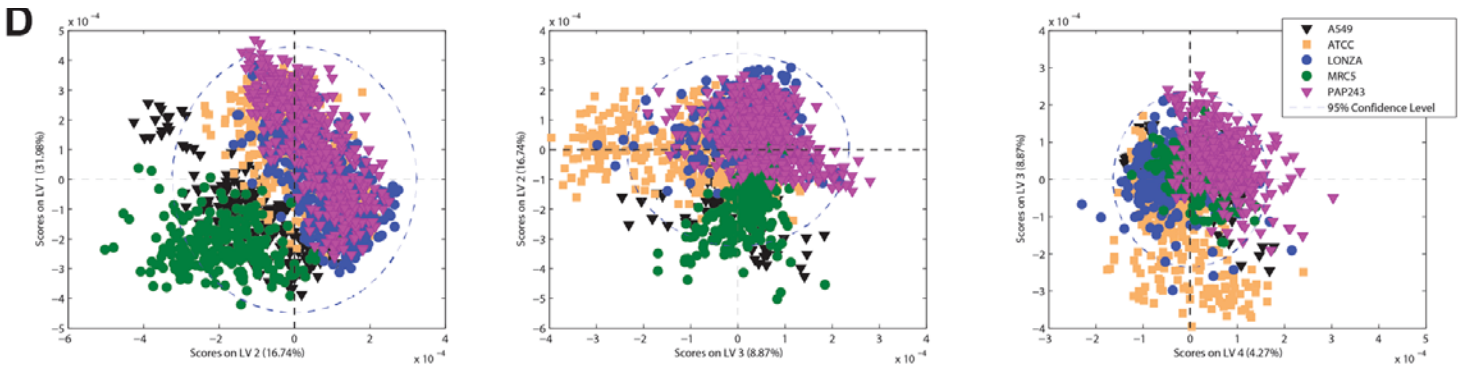
- Patient derived culture shows greater difference to the commercial cultures





Testing the classification potential for HBEC & immortalized cell line cultures

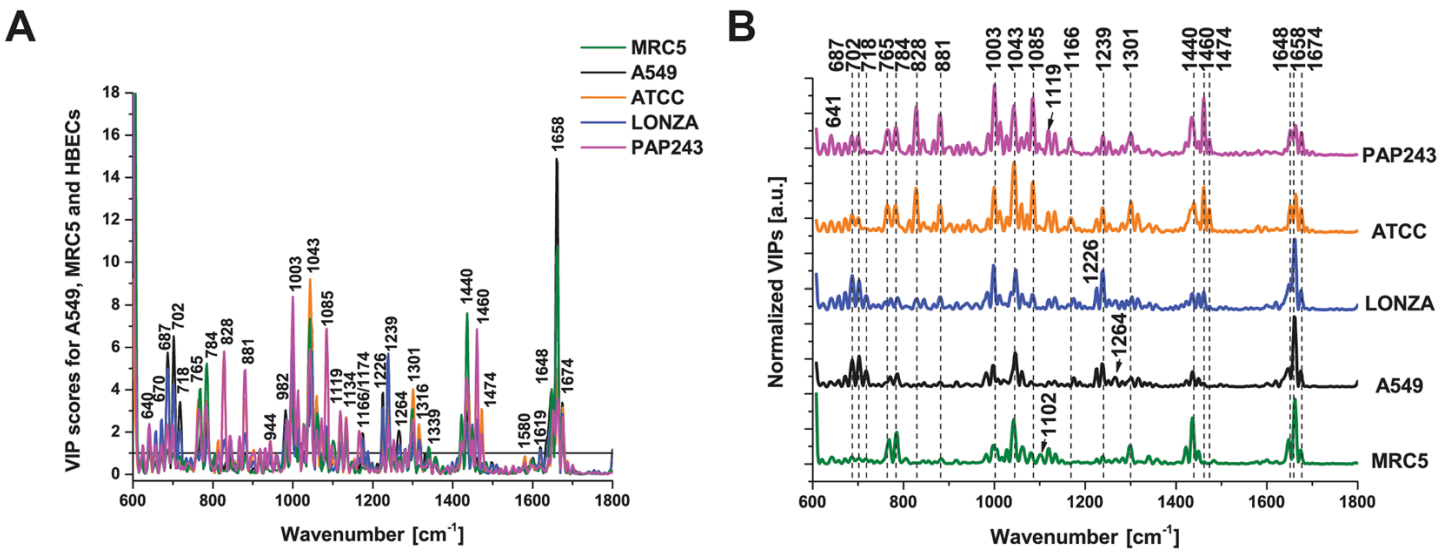
- Partial least squares discriminant analysis (PLS-DA) – a chemometric technique widely used to optimize separation between different sample groups
  - Up to 4 latent variables show good discrimination
- Classification of average line-scan spectra: sensitivity, specificity, area under the curve
- Further performance confirmation using
  - root mean square error of calibration,
  - - of cross-validation and
  - – of prediction
  - → good validity cirteria using PLS-DA



Cell line	Sensitivity	Specificity	AUC	RMSEC	RMSECV	RMSEP
MRC5	100%	98.2%	0.9897	0.1328	0.1462	0.1614
A549	100%	94.6%	0.9732	0.1352	0.1474	0.1631
HBEC PCS-300–010 (LONZA)	93.8%	91.7%	0.9648	0.2210	0.2450	0.2628
HBEC CC-2440 (ATCC)	93.8%	97.9%	0.9767	0.2064	0.2294	0.2138
HBEC PAP243	93.8%	93.8%	0.9831	0.2282	0.2454	0.2426

Variable Importance in Projection (VIP) scores for PLS-DA model of all Raman data

- To identify most discriminatory spectral regions for immortalized cancer cells, fibroblasts and HBECs
  - Lipids: 718, 1264, 1301, 1440, 1658
  - Proteins: 641, 1003, 1166/1174, 1239, 1580, 1658, 1674
  - Nucleic acids: 784, 828, 1316, 1458
  - Carbohydrates: 881, 944, 1034, 1085



Conclusions & Significance

- Development and application of an optimized protocol for Raman micro-spectroscopy of different living human lung cell lines in culture
- Successful delineation of immortalized cell lines mainly based on differences in lipid composition
- PLS-DA model performance of all 5 cell types investigated
  - Highly relevant Raman bands: lipids, proteins, nucleic acids and carbohydrates
  - high average sensitivity of 96.3% and specificity of 95.2%
- Raman features associated with lipids might be useful as biomarkers for monitoring cancer progression
- Probably useful in identifying different primary HBEC cultures to identify different lung cell types in co-cultures and study early carcinogenesis

Thank you for your interest!

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