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Label-free Digital Pathology

Diagnosis and Biomarker Research

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Gold standard today for cancer diagnosis in the clinic is the visual inspection on H&E labelled tissue thin sections by a pathologist. The presented “label-free digital pathology” approach uses spatially resolved measured vibrational spectra as fingerprints for the biochemical status and respective the spectra are labelled automatically observer independent by bioinformatics.

In addition to H&E staining immunohistochemical (IHC) stains are used in which single specific molecules act as markers for disease. Proper evaluation depends on reliable, reproducible staining and the pathologist's expertise. We established a label-free and inter-/intra-observer-independent approach for classification of tissue thin sections by (FT)IR (Fourier transform infrared) imaging. In this new approach, the unstained tissue is imaged with an infrared microscope and the “IR image” of the tissue is classified bioinformatically. The resulting index color images represent the tissue classification including cancer type, subtypes, all tissue types, inflammation status and even the tumor grading. [1-3] All this can be resolved without labeling and without inter-/intra-observer variability. Besides its application in diagnostics, it can be combined with omics techniques to provide molecular resolution. After label-free tissue classification, the spatially very resolved tumor region can be cut out with laser microdissection and the sample can be subsequently analyzed by different omics techniques. We recently show these exemplary for subtypes of diffuse malignant pleural mesotheliomas which were

subsequently analyzed with proteomics. [4] Mesothelioma tumors are mainly caused by asbestos. This combines label-free spatial resolution with a molecular resolving method. Thus, the differently expressed proteins in the two subtypes can be identified. A detailed bioinformatical analysis then selects the biomarker candidates from the larger number of identified proteins. In this approach, all biomarkers of clinical immunohistochemistry used today for mesotheliomas could already be identified on a small number of test persons. This validated this new approach. In order to reduce the measuring time instead of time consuming FTIR imaging a quantum cascade laser (QCL) based IR microscope is used. In a pioneering study we show for the first time that the QCL-based IR imaging classifies exemplarily colorectal cancer as compared to a pathologist with specificity and sensitivity >94% in the same time range of few minutes for a fresh frozen thin section.

What Is FTIR imaging?

In FTIR imaging for label-free tissue diagnostics, we record spatially resolved vi-

brational spectra of a tissue thin section using an infrared microscope. A vibration spectrum reflects the biochemical status of the proteome, genome, transcriptome, lipidome and metabolome in a tissue at the pixel measured. Alterations induced by cancer are reflected in the altered spectrum. The spectrum is thus representative of the status of the sample, just like a fingerprint is characteristic of an individual person. Approximately twenty million infrared spectra are usually recorded to produce one single tissue image. Using bioinformatics image analysis, the spectra are classified by a random forest (RF) classifier trained with a reference database. This database contains spectra of already known tissues and tumors, and has been established as collaboration between pathologists, biophysicists and bioinformaticians. The analytical program allocates each spectrum to tissue types that have been stored in the database, represented by a specific color — just like an offender who can be identified by comparing his fingerprints with previous database entries. This produces a spatially resolved annotated image of the tissue section. The process is label-free and the tissue sample is not altered. In 2013 and 2015, we were able to demonstrate the potential of FTIR imaging for colorectal cancer diagnostics with a sensitivity of 94% and a specificity of 100% and for the characterization of thoracic tumors with a sensitivity of 91% and a specificity of 97% compared to histological annotation. The

differential diagnosis of the subtypes of adenocarcinoma of the lung was achieved with an accuracy of 96%. [1,2] Driven by the desire to combine very precise label-free spatially resolved tissue classification by FTIR imaging with molecular data, we have developed the method of FTIR-coupled laser microdissection.

(FT)IR-coupled Laser Capture Microdissection (LCM)

In order to obtain label-free tissue samples from thin sections for further investigations, the spatially resolved tissue classification obtained is transferred to an LCM. The LCM is a microscope that can cut the tissue with a UV laser and thus enables the targeted collection of selected tissue areas. This is traditionally carried out on subsequent cuts of colored thin sections. However, the tumor distribution can be different in the adjacent section. No homogeneous sample of a certain tumor is then obtained. The newly developed method replaces the step of staining with label-free imaging and thus allows direct collection on the same section for native tissue thin sections. The tissue is characterized with (FT)IR imaging using the tissue-specific spectra. The

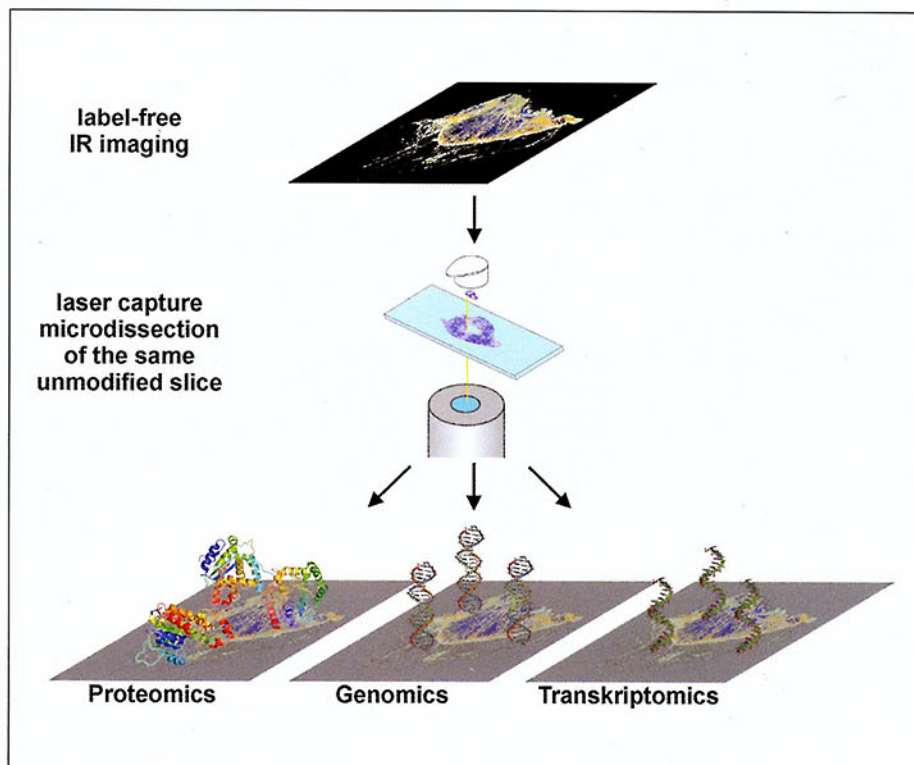


Fig. 1: The label-free characterized tissue thin section can be subsequently cut with laser capture microdissection based on regions of interest (ROI) obtained from the IR imaging analysis. With subsequent omics the spectral homogenous sample can be analyzed on molecular level. This information can then be used for further diagnostics e.g. gene panels or biomarker research for new more precise candidates.

regions of interest (ROI) are then transferred to an LCM, where they are cut out of the same native tissue thin section and collected. These native homogeneous samples are then available for molecular but not spatially resolving analysis methods such as proteomics or genomics (Fig. 1). The approach allows tissue to be characterized very precisely with a high sensitivity and specificity and to be collected for -omic studies, as was shown in one of our current studies using the DMM subtypes. [4]

FTIR Imaging vs IR Imaging

Unfortunately, the FTIR imaging technique is quite slow and the instruments are not easy usable. The actual instruments need up to 2 m² of bench space and liquid nitrogen for the used detectors - not very practicable for use in a clinic. Furthermore, analyzing an area of 4 cm² can take up to 4 days with very good data quality. All this hinders the translation of the technique into the clinics and biomarker research. These issues can be overcome by using the quantum cascade laser (QCL) as high power light source instead of the thermal light source in FTIR set-ups. The Bhargava and Petrich groups developed home-made QCL-based microscopes [6, 7] which show that they can be used in principle but show laser based artefacts like coherence effect. We handle these problems and show that known side effects of the QCL based systems like coherence can be overcome by a well-trained classifier. We showed the first IR imaging system for which the clinical usability was shown for typical clinical used tissue thin slices with sizes up to 2x4 cm. In combination with our new developed classification model of colorectal cancer tissue thin sections this allows spatially resolved, label-free, automated, and inter-/intra-observer/operator-independent annotation within few minutes, the same time range used for a frozen section by pathologists. [5]

IR Imaging for Colorectal Carcinoma

We studied 100 samples with UICC Stage II and III colorectal cancer tissue and 20 tumor-free tissue samples of 110 randomly chosen patients older than 18 years and developed a workflow that enables the tissue classification for diagnosis in about 30 min for large thin sections, while smaller regions of interest can be analyzed within few minutes both with a sensitivity of 96% and specificity of 100% as compared to histopathology (Fig. 2).

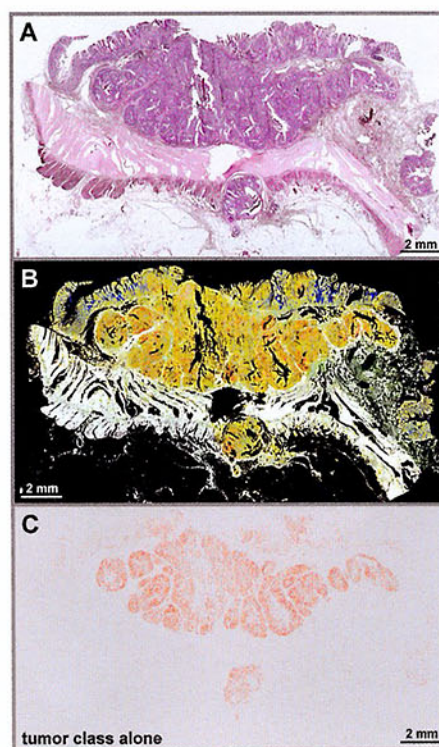


Fig. 2: Images of whole slice colorectal cancer tissue thin sections. Thin tissue section images of colorectal cancer marked by H&E in A and label-free IR images in B and C taken by QCL based IR microscope and analyzed by an improved new classifier. (A) H&E stained image of colorectal cancer thin section, showing morphological structures of diseased colon wall. Cancerous regions are prominently illustrated in the upper part by heavy purple hematoxylin staining patterns. Below is the muscularis propria surrounded by connective tissue. Infiltrating inflammatory cells are distributed over the whole tissue sample. (B) IR image of the classifier with the following tissue classes: red: pathological region; yellow: infiltrating inflammatory cells; white: muscle; green: connective tissue; cyan: crypts; blue: lumen. The morphological architecture is well represented by the index colors and agrees nicely with the annotation of a clinical pathologist. (C) IR image showing the tumor class identified in the second classifier. The red pixels represent the cancerous region of the sample matching the annotation by the clinical pathologist in A. Figure reprinted from Kuepper, Kallenbach-Thieltges et al., Scientific Reports 8, Article number: 7717 (2018)

As control, the measurements were carried out using two IR imaging systems, and the analyses were performed by several users; this did not affect the results. Therefore, the method is now very fast, reliable and does not depend on a specific device or a specific user. This opens up new avenues for automated classification of tissue samples taken directly from the patient. In future, we intend to incorporate the method into clinical workflow. The automated image analysis might be deployed as a time-saving diagnostic tool, which might possibly even be used in-situ.

Colorectal cancer is one of the most common tumor diseases, and it is very treatable following early diagnosis. The results of the study give rise to hope that highly precise therapy is within reach, which can be personalized for each individual patient and, consequently, will ultimately prove more successful than traditional approaches. Furthermore, label-free and observer independent IR imaging tissue characterization might revolutionize the homogenous tissue collection for biomarker research.

Conclusion

An automated and label-free approach is established which classifies tissue thin sections in the same time range as a

pathological diagnosis and with high sensitivity and specificity >94% as compared to pathological diagnosis. Furthermore, it is possible to select very homogeneous samples for the biomarker search by integration of IR-coupled LCM for subsequent omics analysis. A transfer to further omic techniques besides proteomics is easily possible. The newly developed approach will pave the way for precise diagnostics and more specific biomarkers that can be used in precision medicine.

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