Functional live-cell mid-infrared microscopy and spectroscopy by optoacoustic and optothermal detection

Tao Yuan[1,2], Francesca Gasparin[1,2], Nasire Uluc[1,2], Vasilis Ntziachristos[1,2], Miguel A. Pleitez*[1,2,]

[1]Technical University of Munich, School of Medicine, Center for Translational Cancer Research (TranslaTUM), Chair of Biological Imaging, D-81675, Munich, Germany
[2]Helmholtz Zentrum München (GmbH), Institute of Biological and Medical Imaging, D-85764, Neuherberg, Germany

ABSTRACT

Mid-infrared (mid-IR) spectroscopy and optoacoustic/optothermal (OA/OT) imaging are perfectly complementary technologies to each other. Vibrational molecular excitations by mid-IR absorption are utterly de-excited in the form of heat while efficient OA/OT signal generation primarily depends on heat deposition. This synergy allows overcoming the (otherwise) persistent limitations of traditional mid-IR spectroscopy and imaging in live-cell/fresh-tissue applications—i.e., sample opacity due to water absorption. Combination of mid-IR excitation and OA/OT detection has resulted in new tools for label-free live-cell, tissues, and in vivo metabolic research. Here we discuss basic principles on mid-IR detection for spectroscopy and imaging as well as the most recent developments on mid-IR OA and OT microscopy that overcome the limitations of conventional vibrational spectroscopy for biosensing and label-free metabolic microscopy.

Keywords: live-cell mid-infrared microscopy, optoacoustic/optothermal detection, label-free chemical microscopy, metabolic imaging, positive contrast detection.

1. INTRODUCTION

Our understanding of cell and tissue biology in health and disease is greatly derived from observations with optical microscopy. However, the strengths of modern optical microscopy greatly rely on the use of exogenous labels such as fluorescent proteins or small-molecule dyes. The application of labels comes with inherent challenges such as risk of perturbation of biological processes, bio-distribution of the label, and (in many cases) its restriction to fixed cells and fixed tissues only. Therefore, label-free metabolic imaging in living cells and tissues is highly desired—but, it remains challenging to conventional optical microscopy (particularly below 700 nm) due to its lack of endogenous chemical specificity and the risk of photodamage.

Vibrational imaging modalities, such as Stimulated Raman Scattering (SRS) and mid-infrared (mid-IR) microspectroscopy, provide chemical contrast and have enormously extended the range of possibilities for label-free metabolic imaging.[1] Nevertheless, besides the risk of photodamage, the detection limit of Raman imaging is above 1 mM; thus, reducing applications in live-cell metabolic imaging (particularly in the fingerprint region) where sub-µM concentrations need to be measured. Mid-IR imaging/spectroscopy, on the other hand, provides higher sensitivity with cross-sections up to eight orders of magnitude larger than Raman imaging. However, the sensitivity and signal-to-noise ratio (SNR) of current mid-IR microscopy and spectroscopy remains inadequate for live-cell metabolic imaging due to the negative contrast scheme of conventional optical detection methods—wherein only remaining (transmitted or trans-reflected) photons are detected. Conventional optical detection exemplifies a paradox in optical microscopy and spectroscopy where: the higher the optical absorption of an analyte of interest, the lower the number of photons that remain available for detection—resulting in reduced SNR and, consequently, low sensitivity. For conventional mid-IR imaging methods, highly absorbing samples such as biological tissues appear opaque. Sample opacity to mid-IR radiation have notably reduced applicability of live-cell mid-IR microscopy to study native (unperturbed) cell metabolism—i.e., without the use of labels.

Here we discuss how positive contrast mid-IR microscopy by OA/OT detection allows overcoming the limitation of sample opacity in live-cell and tissue mid-IR microscopy.

*miguel.pleitez@helmholtz-muenchen.de; Translational Optoacoustics Lab

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2. METHODS

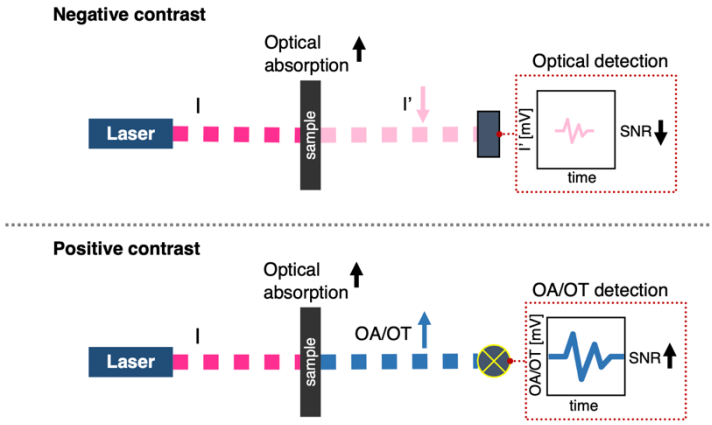

Figure 1: Negative contrast optical detection; the higher the optical absorption, the lower the signal detected. Positive contrast OT/OA detection; the higher the optical absorption, the higher the signal detected.

Positive contrast detection **(Fig. 1)** derives from the synergic action of mid-IR excitation and OA/OT sensing. Contrary to conventional optical detection in positive contrast OT/OA detection the higher the optical absorption, the higher the signal detected; increasing SNR as optical absorption increases. Here we describe two different approaches using positive contrast OA/OT detection for label-free metabolic imaging: **1)** Mid-infraRed Optoacoustic Microscopy (MiROM) and **2)** Wide-field Optothermal Mid-infrared Microscopy (WOMiM). MiROM uses tightly focused optical excitation with coaxially focused ultrasound detection and chemical-contrast imaging is obtained by point-by-point raster scanning the sample along the focal plane; simultaneously acquiring OA signals produced at specific molecular vibrations excited by pulsed mid-IR radiation **(Fig. 2a)**.[2] MiROM circumvents sample's optical opacity in conventional mid-IR microscopy by detecting ultrasound waves instead of mid-IR photons, which—unlike acoustic waves—are strongly attenuated by tissue and water. Contrary to MiROM, WOMiM is a wide-field chemical-contrast imaging method, which uses pump-and-probe detection of OT signals by optical phase change due to mid-IR excitation **(Fig. 3a)** to obtain a wide field-of-view micrograph without the need of raster scanning.[3] Chemical-contrast imaging is obtained by subtraction of two-phase contrast snapshots (with and without mid-IR illumination) **(Fig. 3b-d)**.

3. RESULTS AND DISCUSSION

The unique features resulting from the combination of mid-IR excitation with OA and OT sensing offer a new tool for biomedical research by allowing unperturbed longitudinal monitoring of metabolites in single-cells and tissues—promoting the development of innovative applications. For instance, with MiROM we are able to monitor lipid, protein, and carbohydrate dynamics down to the single-cell level **(Fig. 2b)**. In living adipocytes, we were able to observe the spatio-temporal distribution of carbohydrates used for triglyceride formation during adipogenesis **(Fig. 2c)**.[2] Additionally, MiROM has also demonstrated potential for application in label-free analytic histology of carotid atherosclerosis [4] and, recently, we have applied MiROM to study the metabolic distribution of glucose in mice in vivo, enabling non-invasive glucose detection in microvasculature.[5]

Similarly, pump-and-probe mid-IR OT imaging is a growing field of research in label-free chemical microscopy, that allows going beyond the resolution limit imposed by the long wavelengths used for mid-IR excitation. Additionally, it facilitates hyperspectral imaging (i.e., each pixel in the image comprises a spectrum) with sub-μM resolution and endogenous molecular contrast [6,7]. Using wide-field pump-and-probe mid-IR optothermal imaging with WOMiM, we achieved chemical-contrast in Triglyceride phantoms and hyperspectral imaging in the range of 2950 to 2830 cm^{-1} for field-of-views up to 180 μm in diameter, at imaging speeds up to 1 ms/frame **(Fig. 3e)**—reducing the trade-off (of point-by-point scanning) between imaging time and imaging area to achieve hyperspectral imaging in a broad wavelength range. Next steps include using WOMiM's imaging speed and large field-of-view abilities to monitor lipid metabolism and fast lipid droplet dynamics in adipocytes to study adipogenesis and lipolysis.

Positive contrast OT/OA detection overcomes optical opacity of tissues in conventional mid-IR detection making possible applications to living cells without restriction of sample thickness and with minimal sample preparation.

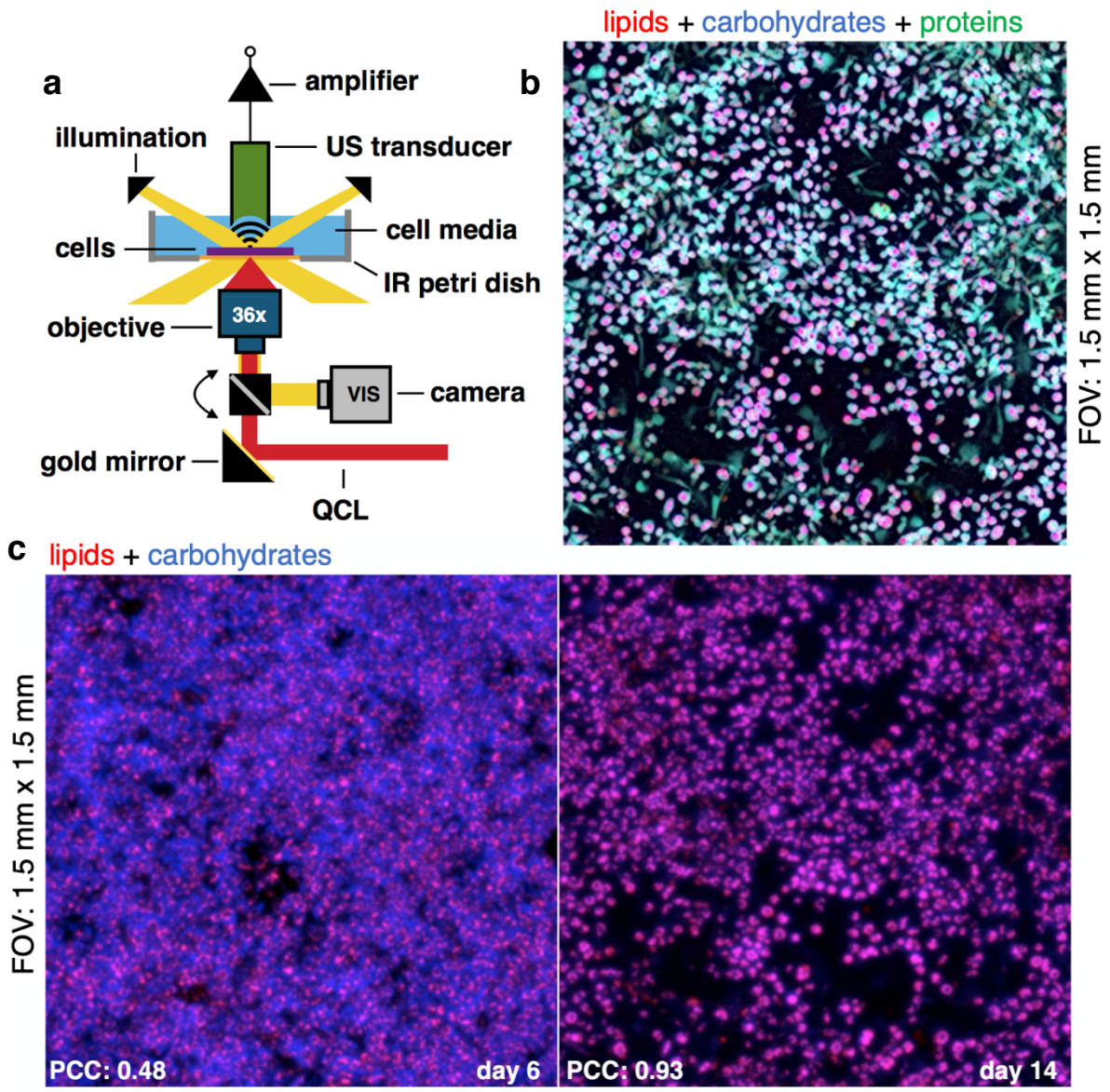

Figure 2: a) Schematic representation of MiROM. **b)** MiROM image of differentiated white adipocytes after 14 days incubation. The figure shows intrinsic biomolecular contrast distributed in living cells. Lipid contrast obtained at 2850 cm^{-1} (CH2 vibrations), protein contrast at 1550 cm^{-1} (Amide II), and carbohydrate contrast at 1022 cm^{-1} (CO/COH vibrations). Due to lipid-carbohydrate co-localization during adipogenesis, lipid droplets appear magenta. Pixel size: 5 µm. **c)** Co-localization of lipid and carbohydrate contrast in adipocytes. At incubation day 6, the carbohydrate contrast is broadly spread around LDs formation, while at incubation day 14 it is highly co-localized with mature LDs. The Pearson Correlation Coefficient (PCC) between channels and confirms this observation. Modified from [2].

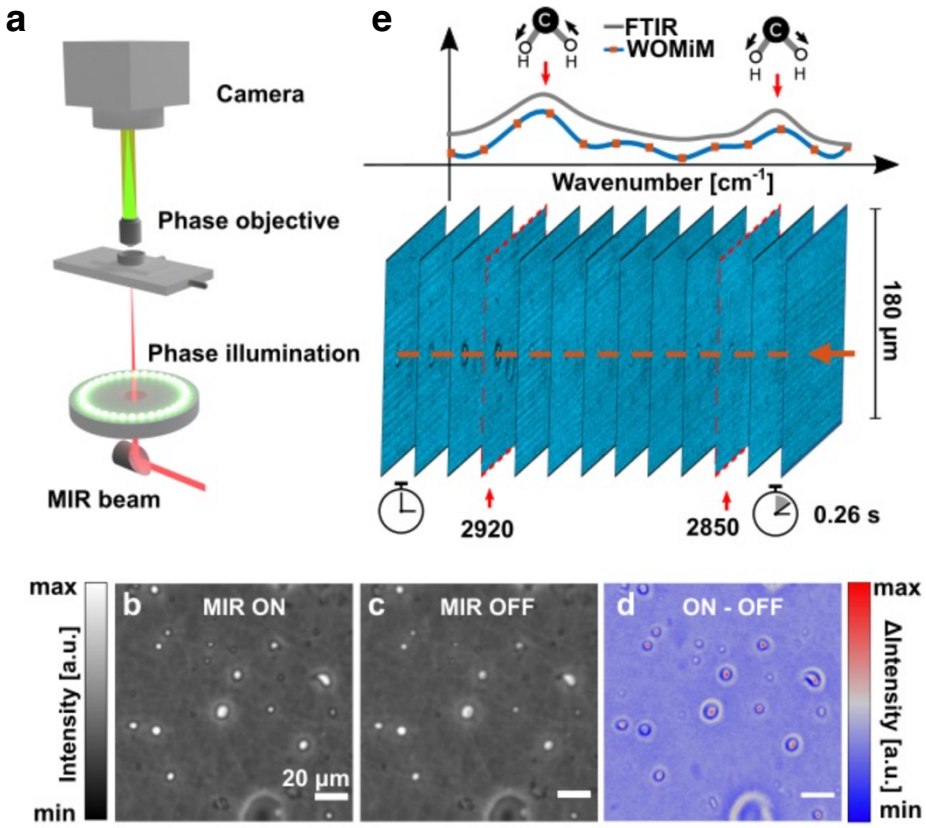

Figure 3: Operational principle and hyperspectral imaging of Triglyceride phantoms by WOMiM. **a)** Schematic representation of WOMiM. **(b)** A MIR-ON image (MIR wavenumber at 2850 cm^{-1}) and **(c)** a MIR-OFF image of Triglyceride phantom. Subtracting the MIR-OFF image (c) from the MIR-ON image (b) yields a subtraction-image **(d)** representing a single WOMiM image at 2850 cm^{-1}. **e)** Hyperspectral imaging on Triglyceride phantoms compared to FTIR spectroscopy. Modified from.[3]

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