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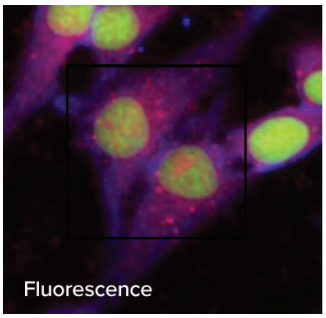
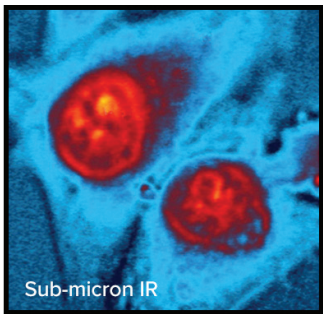
Life science applications of sub-500nm IR microscopy and spectroscopy with co-located fluorescence imaging

Advances in life sciences research and technologies are transforming health around the globe and have the potential to provide innovative, new and improved ways to support a healthier worldwide population.

O-PTIR microscopy and spectroscopy offers significant advantages for life science applications:

- Highest resolution IR microscopy and spectroscopy achieving <500nm resolution on biomolecular structures providing the highest resolution investigation of molecular chemical structures
- Simultaneous IR and Raman microscopy provides complementary and confirmatory results with measurements at the same spot at the same time with the same resolution
- Co-located with fluorescence microscopy to quickly identify biomolecular structures of interest and subsequent sub-500nm IR resolution microscopy and spectroscopy
- The O-PTIR technique enables both single cell and multi-cell analysis for broader research studies and understanding of cellular processes





O-PTIR has overcome many of the fundamental limitations of conventional IR spectroscopy.

→ **These advances drive** increasing government and private investments into life science studies and subsequently drive researchers to supplement their existing analytical and scientific research equipment with new, novel techniques, models, and algorithms to support these scientific breakthroughs.

The life sciences comprise fields of science involving the study of living organisms such as plants, animals, and humans. Fundamental to supporting many of these new discoveries is understanding the chemical molecular structure of cellular and bio-molecular components and processes.

Many techniques exist to investigate these molecular processes and chemical structures. Fluorescence microscopy is an example of a critical technique central to life science research, providing high molecular specificity in a broad range of application areas. However, fluorescence is limited in its ability to provide chemical molecular structure.

Figure 1: The mIRage-LS Sub-500nm IR multimodal microscope

Infrared (IR) and Raman spectroscopy and imaging systems, are well established techniques in life science research, providing broad macromolecular, spatially resolved characterization capabilities that have been applied to a host of application areas including studying the biochemical environment of single cells or monitoring the reaction of cells to drugs, and for use in the pharmaceutical industry for process and quality control in the manufacturing of drugs.

However, conventional IR is limited in its spatial resolution for life science and Raman microscopy is limited in its use with fluorescence microscopy due to fluorescence interference issues. A novel new approach, Optical Photothermal Infrared (O-PTIR) spectroscopy, has overcome many of the fundamental limitations of conventional IR for life science applications, while also providing excellent complementarity with Raman spectroscopy and a fully integrated approach with fluorescence microscopy.

This application note describes the novel O-PTIR approach and its integration with Raman and fluorescence techniques for life science applications and highlights a range of high value life science applications.

Novel IR technique Raman and infrared spectroscopy and imaging

Current traditional IR and Raman spectroscopy techniques, while having certain advantageous properties, also have their respective limitations which hinder the broader penetration of these techniques into mainstream life science research. For example, key limitations of IR spectroscopy include limited spatial resolution (~10-20 micrometers), strong water absorbances limiting live cell studies and spectral distortions (Mie scattering) with spectra often being dependent on sample morphology, in addition to the sample chemistry. With Raman spectroscopy, while it has excellent spatial resolution (<1 micron) enabling sub-cellular resolution and is water compatible, it often suffers from autofluorescence issues which can swamp the weak Raman signal. Also, being an inherently weak phenomenon, Raman often suffers from poor signal-to-noise ratio necessitating long measurement times.

The recent advent of O-PTIR spectroscopy, with its submicrometer and simultaneous Raman capabilities, O-PTIR has overcome the issues that have plagued traditional IR and Raman spectroscopy, thus now delivering submicrometer IR spatial resolution, without scattering artifacts, is water compatible with the ability to also couple in Raman to delivery IR+Raman spectroscopy at the same time, with the same spatial resolution, from the same spot. This allows for the first time, the synergistic exploitation of the true chemical complementaries of IR and Raman. These techniques can deliver broad macromolecular characterization and can now be performed on biologically relevant spatial scales, <500nm, allowing uniquely for IR spectroscopy, sub-cellular resolution, that is matched with Raman and fluorescence imaging resolution.

Now, for the first time, with the mIRage-LS product the integration of widefield epi-fluorescence imaging into this submicrometer simultaneous IR+Raman platform enables the sample registration free combination of these techniques into a single instrument heralding a breakthrough for life science research, allowing researchers to truly exploit these two techniques with powerful synergy, to access additional information and insights not available with either technique on its own while aligning with existing life science workflows. Additionally, with the introduction of the new counter-propagating mode, improved IR spatial resolution is achieved to open up new applications for O-PTIR spectroscopy.

Breaking the IR spatial resolution barrier with O-PTIR

The infrared spectroscopy spatial resolution limitation has been overcome with the development of O-PTIR microspectroscopy, making sub-micrometer spatial resolution and high resolution chemical structural imaging and spectroscopy of a variety of cellular and sub-cellular structures a reality with IR.

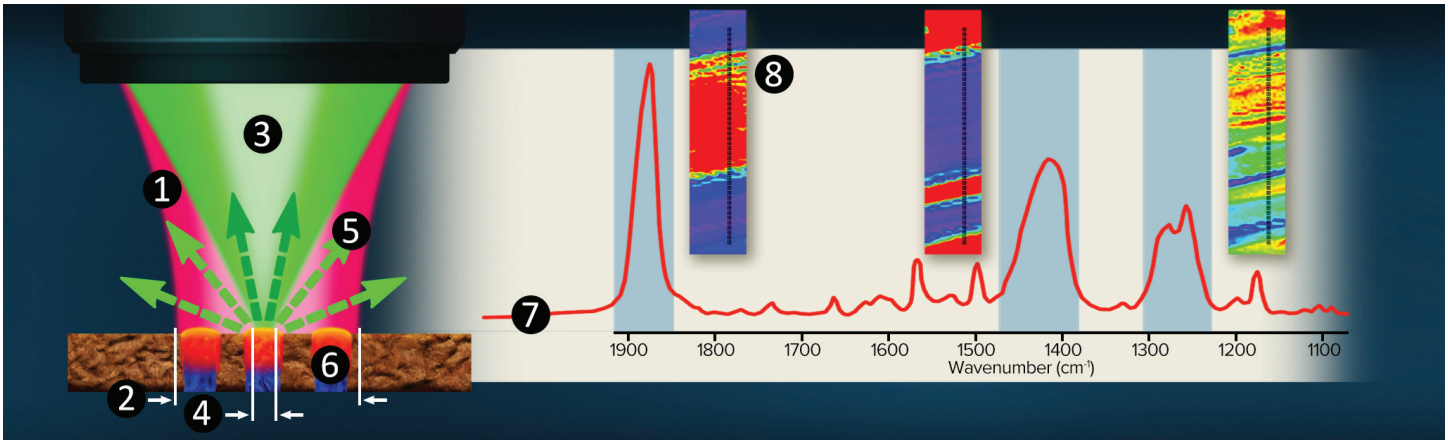


Figure 2: O-PTIR Co-propagating technique overview

O-PTIR reflection mode co-propagating technique

The technique as described is illustrated in figure 2.

1. A pulsed, tunable, infrared laser source (pump) is focused through a reflective (Cassegrain) objective.
2. The infrared diffraction limit is 10-20µm, thus it cannot resolve the small blue inclusions vs brown.
3. A colinearly introduced visible laser, typically a 532nm green Raman excitation laser (probe), is focused through same objective.
4. The shorter wavelength green laser can be focused thirty times better than IR and can focus on just the blue region.
5. The green reflected (or transmitted) laser is detected as a function or IR laser wavelength tuning.
6. When the infrared wavelength matches and absorption band in the sample the energy is absorbed causing a photothermal response in the sample, thus changing the green reflectivity.
7. Synchronously monitoring the green laser response while tuning the IR source, creates an infrared spectral response.
8. Fixed frequency imaging or hyperspectral data collections are available for mapping chemical functional group distributions.

Infrared spectra are measured via indirect detection of a reflected (or transmitted) 532nm visible laser leading to submicron IR spatial resolution. Although single spectral scans can be collected in less than a second, a scan of approximately twenty seconds for each sample location generates an IR spectrum with excellent signal-to-noise ratio.

O-PTIR counter propagating technique

An additional major innovation with the mIRage-LS platform is the addition of a new high spatial resolution IR mode, termed "counter-propagating" mode as seen in figure 3. The key enabler of higher spatial resolution is the ability to utilize regular high power glass objectives as the probe collection optic. Unlike "co-propagating" mode where all-reflective Cassegrainian optics are used to focus both the IR pump and visible probe beam, the two beams are decoupled with the IR beam directed via the underside of the sample, allowing for high power glass objectives to be used to delivery and collect the probe beam.

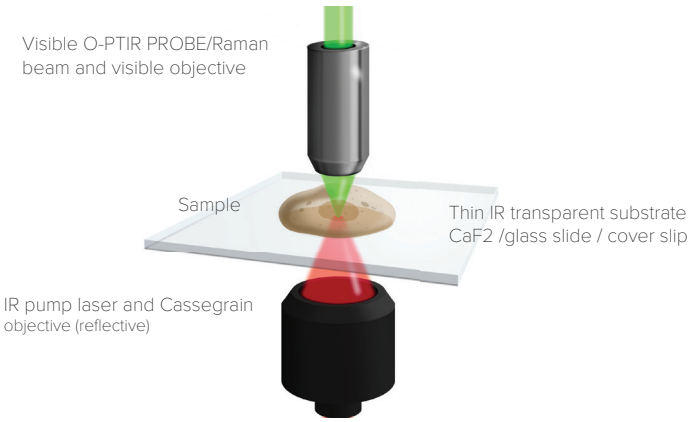


Figure 3: Counter propagating mode. IR light arrives from the bottom and the probe laser measures the sample response through a high NA visible objective

The counter propagating method retains all the benefits of O-PTIR while also providing for increased spatial resolution and greater experimental flexibility, with options now for the use of

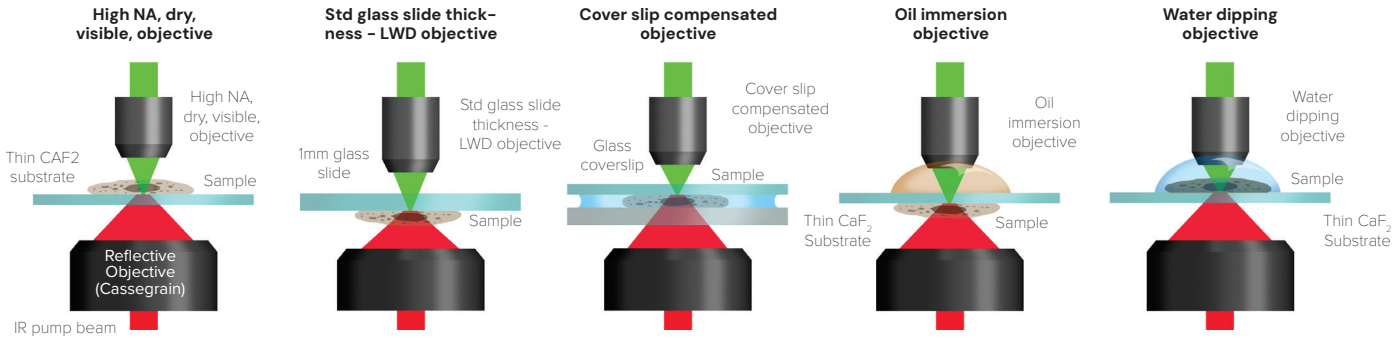


Figure 4: Counter-propagating mode provides application flexibility with a wide range of set ups for sample presentation

water-dipping objectives, oil-immersion objectives, etc. Figure 4 illustrates some of these new modes of operation.

Simultaneous Raman/O-PTIR principal of operation

The unique concept and design, as seen on figure 5, of the mIRage-LS enables Raman and O-PTIR to operate simultaneously on the same spot on the sample, at the same time with the same spatial resolution. With this simultaneous measurement capability, researchers can utilize the complementary nature of both techniques and provide confirmatory spectral information for more accurate measurements.

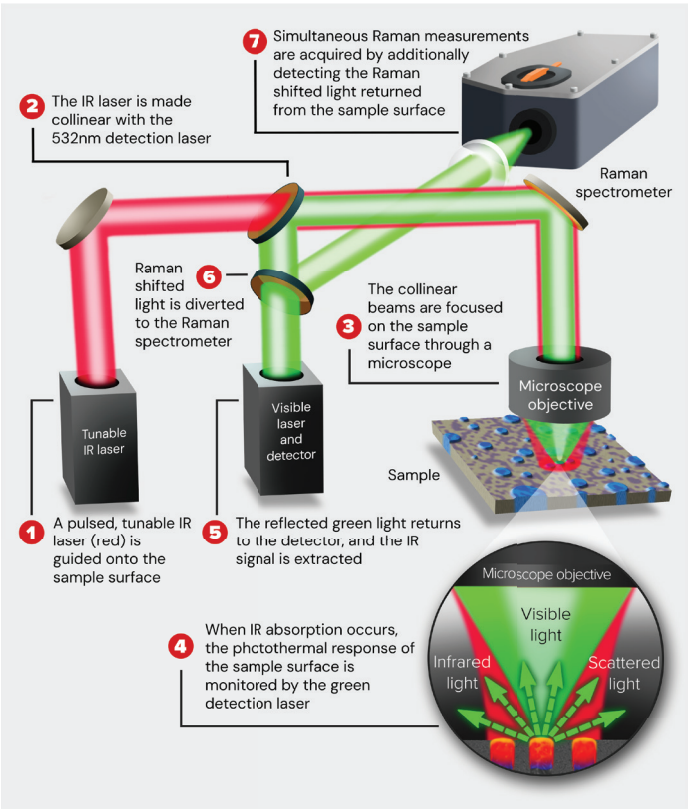


Figure 5: Schematic description of the combined O-PTIR and Raman instrument

Mirage LS principal of operation O-PTIR and fluorescence microscopy

The system layout for a combined O-PTIR sub-micrometer IR microscope, co-located with fluorescence microscopy is illustrated in figure 6. In counter propagating mode, the

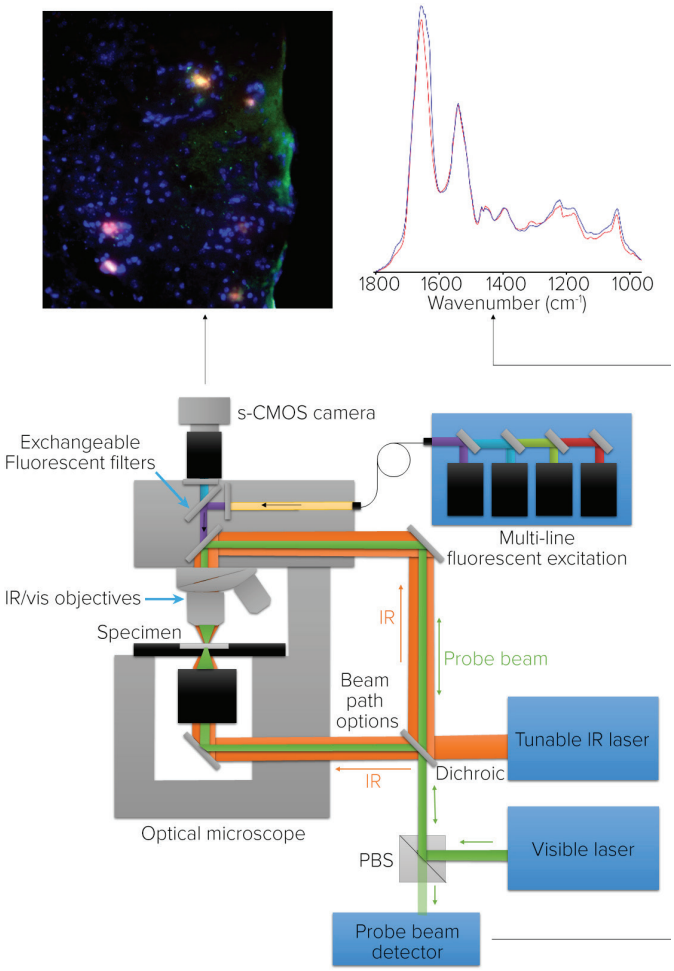


Figure 6: Microscope schematic for O-PTIR and co-located fluorescence microscopy

pulsed IR beam is directed through the underside of the sample. The fluorescence and visible probe beam paths are co-linear through the microscope objective enabling co-located measurements. The fluorescence microscope is used for identifying the biomolecules of interest with high molecular specificity and O-PTIR measures those same features with high resolution IR spectroscopy and imaging. The fluorescence camera includes an illumination unit with a brightfield filter cube and capacity for seven other filter cubes to support a broad range of fluorescence applications.

Cellular, protein and tissue applications with O-PTIR and combined techniques

Neuroglioma cells characterization using counter-propagating mode

Neuroglioma cells were stained with G3BP1 for protein stress granules, DAPI for nucleus and BODIPY for lipids, as shown in figure 7.

On the top left is an RBG overlay widefield epi-fluorescence image with red showing protein stress granules, blue showing nucleus and green showing lipids. Square markers show locations of O-PTIR spectral collection.

On the top right is the brightfield image. Square markers show locations of O-PTIR spectral collection.

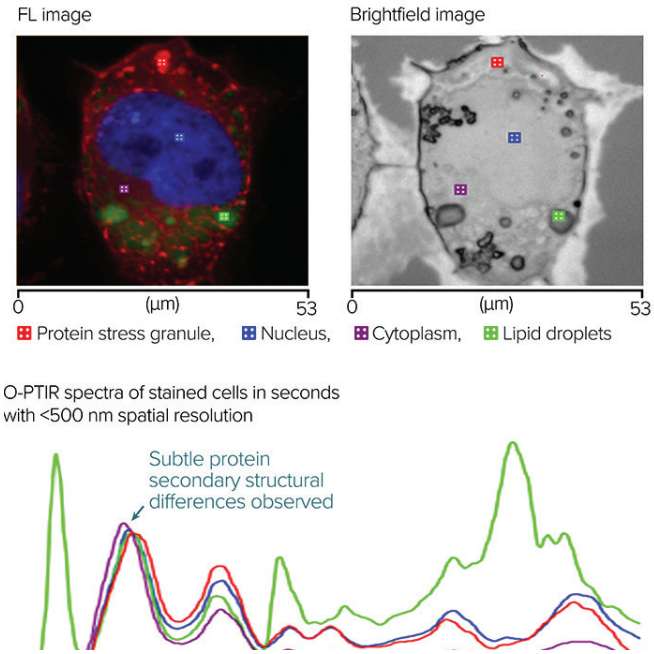


Figure 7: Fluorescence guided Stained Neuroglioma cells and corresponding O-PTIR spectra

On the bottom is the O-PTIR spectra that was collected in seconds from the marker locations shown in the left and middle panes. Clear spectral differences can be observed, consistent with the targeted sub-cellular features. Of particular note, is the subtle shift in the Amide I band of the protein stress granule indicating a likely different protein secondary structure from the other locations.

Blood cells – monolayer measurement using IR + Raman

IR+Raman analysis of a monolayer of red blood cells collected in co-propogating mode off the surface of a glass microscope slide as shown in figure 8. The top left side shows an optical image that is a 70µm x 70µm region where a Raman image, on the top right, was subsequently collected at 1583cm^{-1} using 532nm excitation. The individual O-PTIR and Raman spectra on the bottom were collected from a selected red blood cell (sub-micrometer resolution). This example shows in a dramatic way the complementarity of IR and Raman spectroscopy. The O-PTIR spectrum is dominated by the hemoglobin protein bands, while the resonance-enhanced porphyrin ring vibrations of the heme group dominate the Raman spectrum.

Tissue measurement with O-PTIR and co-located fluorescence microscopy

An Alzheimer's disease mouse model brain tissue section was stained with Amytracker 630 to highlight amyloid

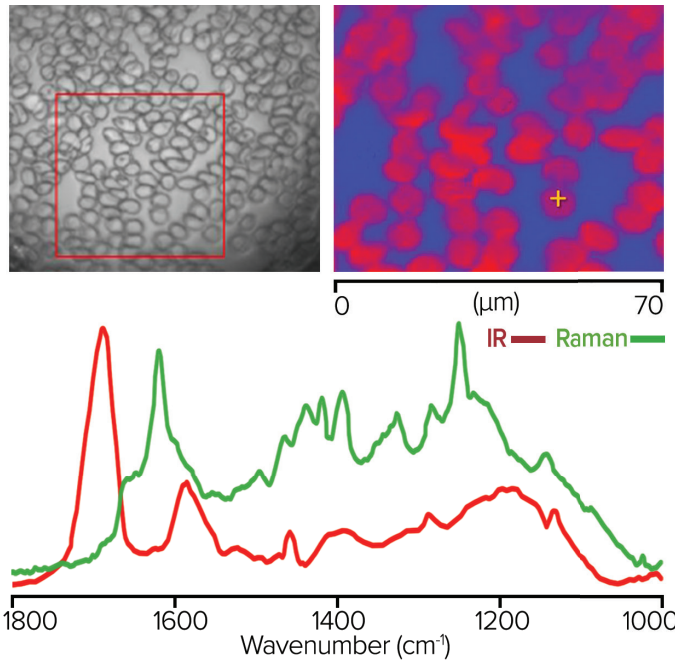


Figure 8: Monolayer of red blood cells , with Raman image and corresponding O-PTIR and Raman spectra

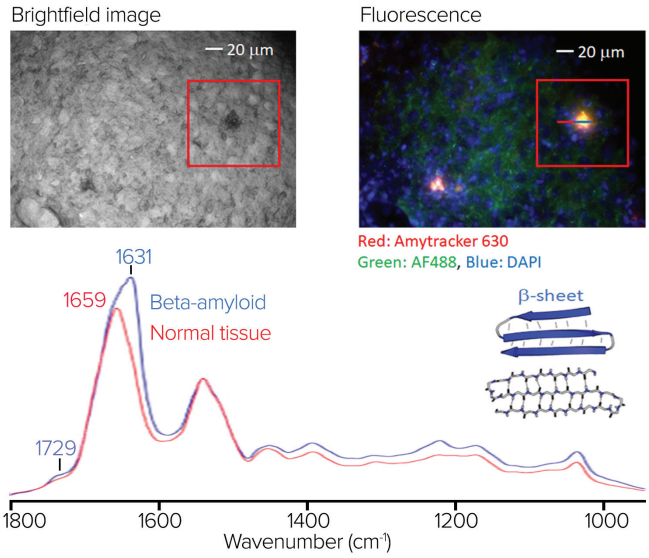


Figure 9: Stained tissue sample with fluorescence image and spectra showing significant spectral differences at 1631cm^{-1}

aggregates, AF488 to highlight proteins and DAPI for the nucleus.

In the top left of figure 9 is shown a brightfield image of the stained sample.

The top right of figure 9 shows the RBG composite fluorescence image, which highlights in red/orange regions of amyloid aggregation. Note how some amyloid aggregates highlighted in the fluorescence image are not readily distinguishable in the brightfield image.

The bottom of figure 9 shows an averaged O-PTIR spectra, from the line profile indicated in the central pane, with spectra averaged on (in blue) and off (in red) the aggregate. The average spectrum of the aggregate shows distinct spectral differences in the amide I band with a significant spectral feature at 1631cm^{-1}, typical of protein beta sheet structures. This clearly demonstrates the utility of combining fluorescence imaging to highlight regions of amyloid aggregation, some of which cannot be readily seen in brightfield microscopy, with submicrometer O-PTIR spectroscopy which can then provide the molecular compositional information, in this case, being particularly sensitive to protein secondary structure, a characteristic strength of IR spectroscopy.

Saltwater algae

Brightfield and autofluorescence (Ex 650nm, Em 690nm) images where collected with a 50x, 0.8NA objective in epi-fluorescence mode. While the brightfield image is relatively feature-free, the autofluorescence can show a wealth of contrast corresponding to known sub-cellular features, as shown in figure 10.

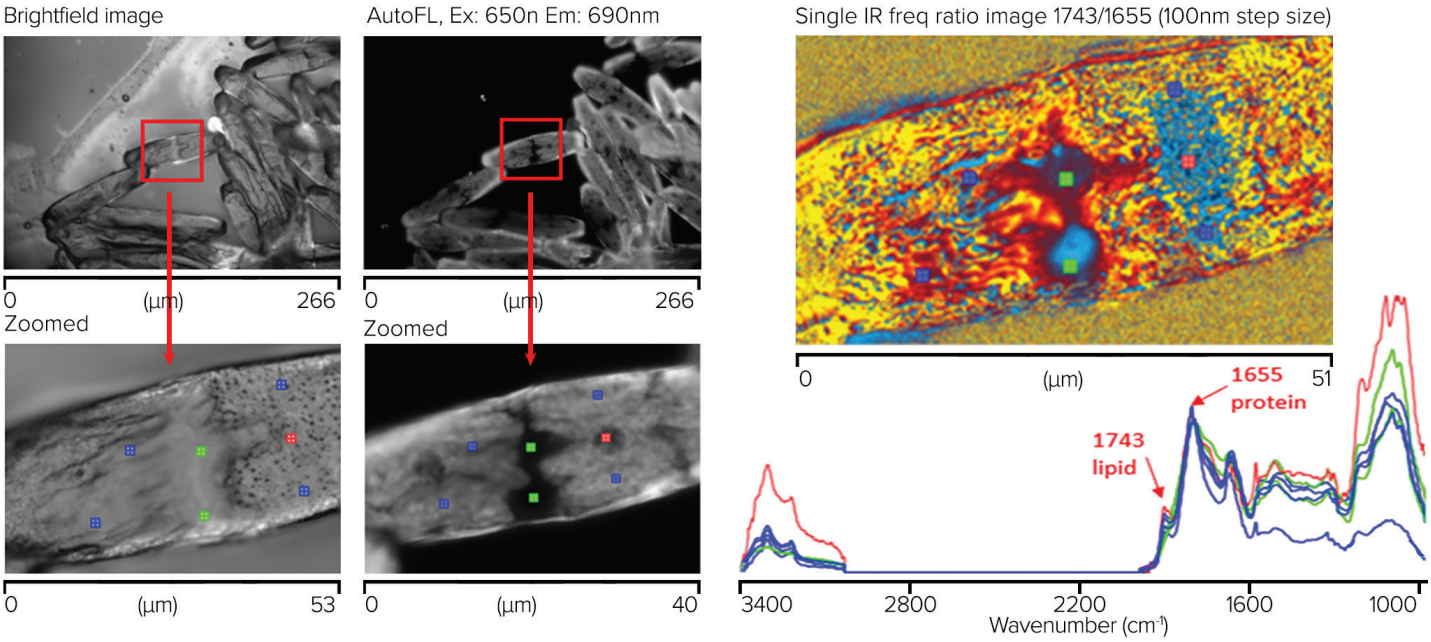


Figure 10: Saltwater algae sample showing measurement locations for corresponding spectra with chemical ratio image (1743/1655cm^{-1})

This autofluorescence image, owing to its higher contrast than the brightfield image, was used to guide the subsequent submicrometer IR and simultaneous Raman measurements. Even though the Raman signals were completely overwhelmed by the sample's autofluorescence, high quality IR spectra, free of any dispersive scattering artifacts (typical of direct IR techniques like FT-IR and QCL microscopy) could be obtained in seconds from ~500nm spots on the samples.

To further explore the macromolecular distributions, a simple lipid-to-protein ratio image was collected at 1743cm-1 and 1655cm-1 for the lipid and protein, respectively. Both the IR image and spectra show distinct features characteristic of this sample, demonstrating the unique potential of this technique to explore such samples for their responses to external perturbations, such as nutrient depletion, changes in nutrients and responses to exposure to chemicals and drugs.

Most of the IR data were collected in the newly developed "counter-propagating" mode, whereby the IR beam is delivered from beneath, allowing the visible (532nm) probe beam to be delivered and collected with high power glass objectives, the same objectives used for brightfield and autofluorescence image, thus ensuring a sample registration free measurement.

Single bacterial cell using simultaneous

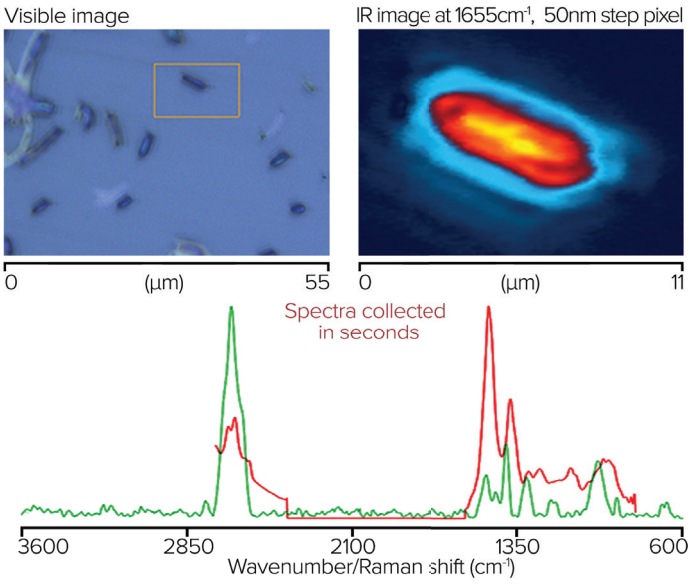


Figure 11: Single bacterial cell, with IR image and corresponding spectra from O-PTIR and Raman

O-PTIR and Raman spectroscopy

Simultaneous O-PTIR and Raman spectra of a single bacterial cell were collected off a CaF2 substrate. A visible image of bacterial cells is shown on the upper left of figure 11. The orange box indicates the region where the single-wavenumber IR image shown at the lower left of figure 11 was collected at 1655cm-1 using a 50nm step size, highlighting the absorption of protein components. The bottom image shows simultaneous submicrometer O-PTIR and Raman spectra collected from the single spot indicated on the single bacterial cell. O-PTIR spectra were collected using a dual range QCL covering the 3000-2700 and 1800-950cm-1 regions in a single unit. No post processing was applied to the O-PTIR spectra and the Raman spectra were baseline corrected.

Fixed cell measurement with oil immersion objective

As a demonstration of the ultimate in optical and IR spatial resolution, an oil-immersion objective (100x, 1.3 NA) was used in counter-propagating mode to measure IR spectral and images of a human cheek cell on an inverted glass coverslip. By collecting molecularly specific single frequency images, in this example at 1740cm^{-1} for lipid, a rapid (minutes) ultra-high resolution image can be collected showing IR features as small as ~285nm, as seen in figure 12. Despite the sample being mounted on glass, spectra are not completely obscured by glass as would be the case in traditional IR techniques such as

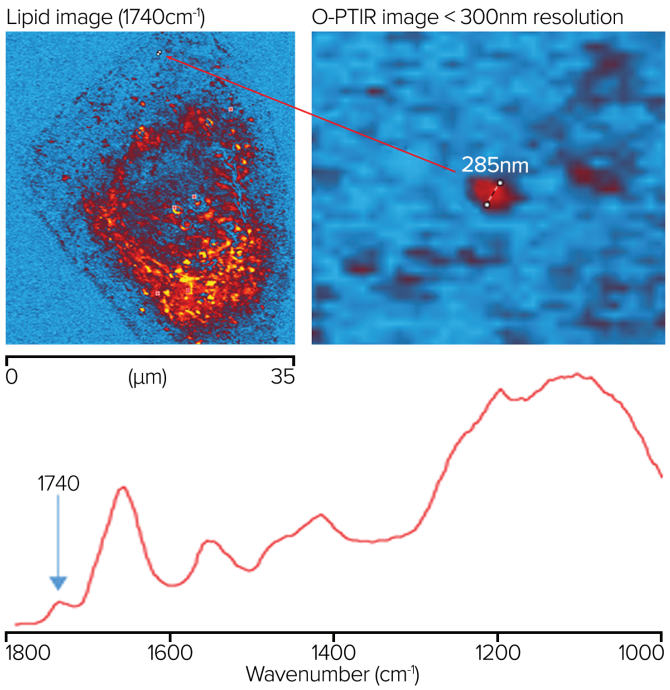


Figure 12: Lipid sample measured at 1740cm^{-1} showing a feature resolution of <300nm with O-PTIR imaging

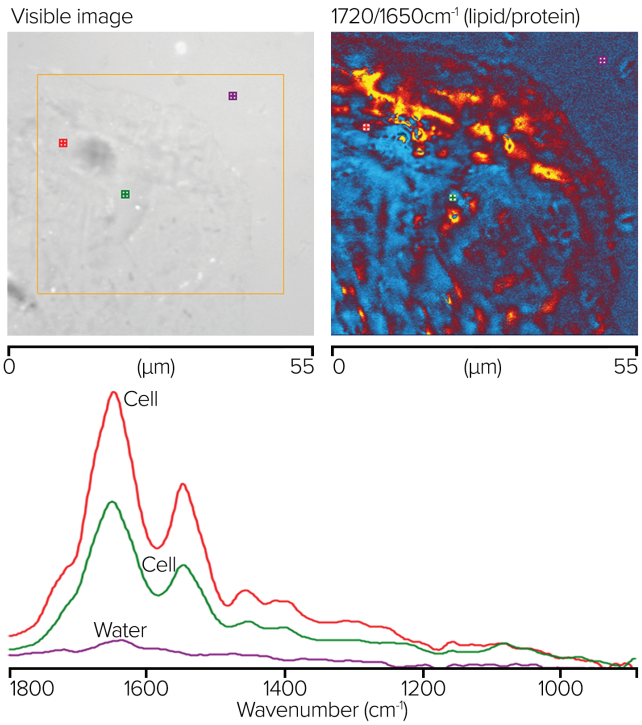


Figure 13: O-PTIR ratio image showing corresponding lipid/protein regions and measured spectra at noted locations

FTIR and QCL microscopy. Instead, a broad silicate feature from the glass is observed, with cellular nucleic acid and carbohydrate features superimposed, while the protein and lipid regions remain completely free of any glass contributions.

Live cells in water with O-PTIR and water dipping objective

Cheek cells were deposited on a 300µm thick CaF2 substrate. A drop of water was deposited on the sample and a visible water dipping objective was used in counterpropagating mode from the top with the pulsed tunable IR QCL source illuminating the sample from the bottom via a reflective Cassegrainian objective (refer back to right side of figure 4). A visible image of the sample is shown on the top left of figure 13. The orange box represents the area where single-wavenumber O-PTIR images were collected at 1720 and 1650cm^{-1} with 50nm spacing. A ratio of these two images is shown at the top right. The red/orange regions of the ratio image represent areas of higher lipid concentration while the blue areas have higher protein concentration. Locations where single point full O-PTIR spectra were collected and the correspondingly colored spectra are shown in the bottom image. No water background compensation was applied, yet spectra exhibit little to no water absorbance.

Conclusion

Several life science applications of O-PTIR microspectroscopy and imaging combined with simultaneous Raman and fluorescence measurements are presented. The mIRage-LS product integrates the simultaneous measurement of submicrometer O-PTIR and Raman spectra with widefield epi-fluorescence imaging, enabling the sample-registration-free combination of these techniques into a single instrument heralding a breakthrough for life science research, allowing researchers to truly exploit these two techniques with powerful synergy, to access additional information and insights not available with either technique on its own while aligning with existing life science workflows.

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