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Microfluidic Approaches to Synchrotron Radiation-Based Fourier Transform Infrared (SR-FTIR) Spectral Microscopy of Living Biosystems

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Abstract: A long-standing desire in biological and biomedical sciences is to be able to probe cellular chemistry as biological processes are happening inside living cells. Synchrotron radiation-based Fourier transform infrared (SR-FTIR) spectral microscopy is a label-free and nondestructive analytical technique that can provide spatiotemporal distributions and relative abundances of biomolecules of a specimen by their characteristic vibrational modes. Despite great progress in recent years, SR-FTIR imaging of living biological systems remains challenging because of the demanding requirements on environmental control and strong infrared absorption of water. To meet this challenge, microfluidic devices have emerged as a method to control the water thickness while providing a hospitable environment to measure cellular processes and responses over many hours or days. This paper will provide an overview of microfluidic device development for SR-FTIR imaging of living biological systems, provide contrast between the various techniques including closed and open-channel designs, and discuss future directions of development within this area. Even as the fundamental science and technological demonstrations develop, other ongoing issues must be addressed; for example, choosing applications whose experimental requirements closely match device capabilities, and developing strategies to efficiently complete the cycle of development. These will require imagination, ingenuity and collaboration.

Keywords: FTIR, live cells, microfabrication, microfluidics, synchrotron radiation.

1. INTRODUCTION

Research within the life sciences is at a defining moment. With an extraordinarily detailed genome-based understanding of cells, many researchers now seek to understand and define the principles guiding translation of the genetic code into the metabolic and regulatory networks underlying cellular function. The recent revolutions in single-cell genomic [1-4], proteomic [5-8], and transcriptomic [9-12] analysis have provided biomolecular details underscoring key processes in biological systems. Modern imaging techniques combining antibody staining or fluorescent markers with computation analysis have allowed researchers to study the spatiotemporal behavior of specific gene products in fixed cells, fixed tissue sections or living cells [13-15]. Complementary to these methods, Fourier transform infrared (FTIR) spectral microscopy (spectromicroscopy or microspectroscopy), a label-free and non-destructive technique, enables real-time acquisition of broadband information on the cellular chemistry [16].

FTIR spectral microscopy uses a combination of visible light microscopy to examine the morphology of a biological specimen and infrared light illumination and interferometer to identify molecular composition. Illumination with infrared light promotes energy exchange between the inherent vibrational modes of molecular bonds and incident photons. These exchanges result in distinct, fingerprint-like spectral bands that appear in absorption spectrum measured as a function of wavelength of incident light (typically expressed in units of wavenumber, cm^{-1}). Figure **1** highlights the origin of different stretching or bending vibrational bands commonly encountered in biological samples. The precise position, line shape, and intensity of these absorption bands depend on the molecular structure and conformation as well as intra- and inter- molecular interactions.

The first experimental demonstration of infrared spectroscopy of biological samples was performed in 1949 using a thermal infrared light source and a dispersive infrared spectrometer approach [17]. In the 1950s, similar approaches were applied for the identification of different species and strains of bacteria [18], to study living muscle cells from insects and animals [19], and for the comparison of spectral features of normal and neoplastic tissues and the chemical constituents (e.g., nucleic acids, carbohydrates, fats and proteins) [20]. Because the measurement process was slow and data analysis was time-consuming, infrared spectral microscopy did not become a widely used tool for studying cellular or tissue systems until the 1990s as a result of three technological breakthroughs: (i) the application of the fast Fourier transform algorithm, (ii) the availability of inexpensive, fast digital computers which enabled the replacement of dispersive spectrometers with FTIR interferometers, and (iii) the introduction of fast-response, high-sensitivity photoconductive single-element mercury cadmium telluride (MCT) detec-

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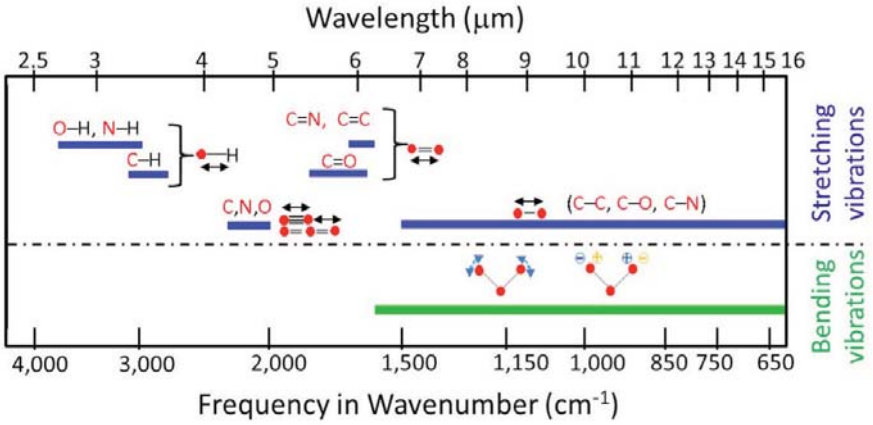

Figure 1. Vibrational modes of common biomolecular bonds - Inherent vibrational motion of molecules gives rise to distinct, fingerprint-like absorption bands in the mid-infrared region. Schematic shows different stretching or bending vibrational bands commonly encountered in biological samples. Infrared spectroscopy is sensitive to the presence of many chemical functional groups (structural fragments) in molecules, and taken together the set of vibration modes are unique for every molecular configuration.

tors. Chemical mapping could be performed by raster scanning the focused beam over the sample and collecting the data using a single point detector. In 1995, Lewis and Levin introduced focal plane array (FPA) detectors with a large number of small photovoltaic MCT detector elements [21] that could be used to image a larger sample region simultaneously. By the 2000s, most FTIR infrared microscopes were capable of using either a single-element detector for mapping or a FPA for imaging.

FTIR spectromicroscopy has emerged as a powerful tool for non-destructive, label-free chemical analysis of the structure and function of macromolecules in complex biological specimens such as cells and tissues. For example, FTIR spectral microscopy has been routinely applied to semi-quantitatively evaluate the relative abundance of carbonate, phosphate and collagen in mineralized bone and cartilage tissues [22], characterize the spatial distribution of main components (i.e., collagen and proteoglycans) in articular cartilage [23-26] and identify age-related structural changes in the DNA of prostate tissues and predict the metastatic state of tumors [27]. It has also been utilized for the detection of microcrystalline deposits of creatine in the brain tissues of post-mortem Alzheimer diseased humans as well as amyloid precursor protein (APP) transgenic mice [28] and for studying the effects of age and diet on the atherosclerotic lesion composition in rabbit aorta [29].

The introduction of synchrotron radiation as a brilliant and broadband infrared source (from far-IR to near-IR) made a significant contribution to modern FTIR spectral microscopy. According to the Rayleigh criterion, the theoretical spatial resolution limit of FTIR microscopes is ~λ, with microscope objectives having a typical numerical aperture (NA) of ~0.5 [30,31]. However, weak signal-to-noise ratio (S/N) provided by conventional thermal IR sources often limits the practical spatial resolution of FTIR microscopes to be ~20-50 μm. In contrast, the infrared light from synchrotron sources is 100-1000 times brighter than that from thermal sources [32], and allows for truly diffraction-limited spatial resolution with excellent S/N when used with a single point MCT detector. The diffraction-limited spot size of 2-10 μm allows mapping the composition and spatial variation of single (or clusters of) cells including microbial [33-36], fungal [37-41], algal [42-44], mammalian [45-51], and even subcellular components of certain mammalian cells [52-54]. Although SR-IR (or S-IR) is a tightly focused source, the low photon energy and low power (compared to IR laser sources) do not introduce detectable biochemical changes within a sample [55]. This non-invasive, label-free approach is greatly beneficial to experimenters that hope to perform further examination of the same sample using complementary methods such as staining [56], proteomic [57], genomic [58], or other –omics based analyses.

With the introduction of synchrotron illumination, interest in imaging live single cells and clusters of cells has increased dramatically. Imaging live cells can reduce some of the artifacts of fixation [59] and real-time FTIR measurements on living biological systems (Figure **2**) are crucial to unravel how their chemical composition evolves with time [43]. This can give insights into many problems of biological or medical interest including metabolic processes [60,61], development [51], and real-time responses to treatment or changing environmental conditions [62,63]. Jamin *et al.* [46] performed the first synchrotron-based FTIR measurements of live cells in 1998. They measured the variation in lipid and protein distributions in mouse hybridoma B cells during cell division and necrosis using a humidified chamber. The use of humidified chambers has continued in a variety of applications including mammalian cell response to polychlorinated aromatic compounds [64], bacterial detoxification of chromium(VI) compounds [65], bacterial degradation of environmental carcinogens [60], oxygen-stress adaptive response in obligate anaerobes [35], and tracking differentiation process in neuron model cells [51]. These chambers, however, do not provide any way to replenish the media and only allow continuous measurement for a few hours before the biological system starts to degrade. Longer-term experiments require either repeated measurements of multiple samples at varying time points, sacrificing both temporal resolution and cell-cell variations within a population, or

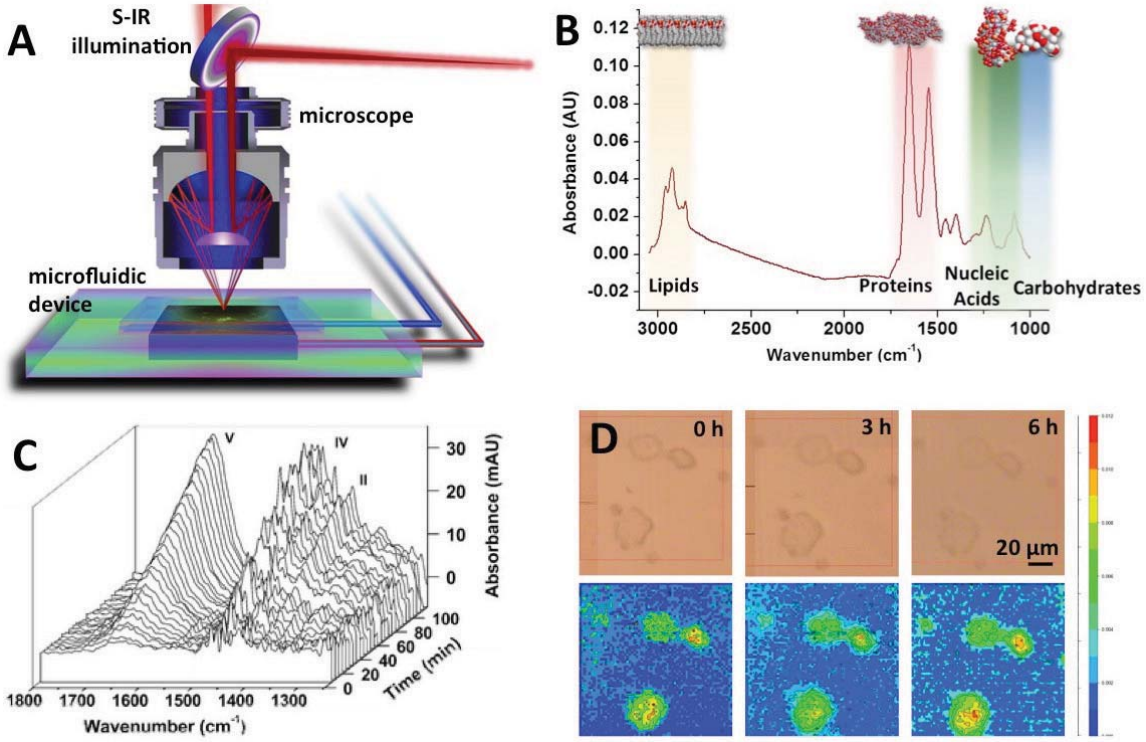

Figure 2. Synchrotron-radiation FTIR of live cells – **A**) Spatiotemporal chemical composition of live cells can be measured in microfluidic devices with an infrared microscope (adapted from Holman *et al.* [16]). Synchrotron infrared light is focused onto the sample by reflective optics and the reflected or transmitted light from the sample is directed to an infrared detector. Environmental conditions are maintained in the device through external fluidic connections. **B**) Infrared spectrum of a single human cell with color bands highlighting absorption peaks associated with constituent macromolecules. **C**) Repeated measurements of the same cells allow cellular processes to be recorded in real-time. Plot shows metabolite formation in single living *Chlamydomonas reinhardtii* (adapted from Goff *et al.* [43]). **D**) Chemical mapping shows how spatial distribution of chemical changes in several cells over time. Upper panels show visible images while lower panels show the lipid distribution in MCF-7 cells at time 0, 3, and 6 hours (adapted from Grenci *et al.* [87]).

alternatively, the use of a microfluidic platform to maintain the biological system viability.

The primary challenge in imaging living biological systems with SR-FTIR illumination is maintaining environmental conditions optimal for cell viability while minimizing the absorption signal from water. Strong IR absorption from the –OH stretching and HOH bending bands can quickly saturate the signal for transmission measurements with water thickness greater than about 10 micrometers [66]. Live cells, meanwhile, consume surrounding nutrients from the media and produce waste that must be exchanged periodically to maintain cell viability.

Despite great progress, SR-FTIR imaging of living biological systems remains challenging because of the demanding requirements on environmental control [16]. To meet this challenge, microfluidic systems have emerged in the last decade as a method to control the water thickness while providing a hospitable environment in order to measure cellular processes and responses over many hours or days. They can be broadly categorized by whether the channel used to confine the cells is closed, where upper and lower IR transparent windows are sandwiched between a spacer, or open, where surface effects are harnessed to maintain a thin water layer at an air-liquid interface. To date, the vast majority of papers on this topic have employed various iterations of closed channel devices while open-channel devices have emerged recently as a viable competitor, albeit with some limitations. This paper will provide an overview of microfluidic device development for FTIR imaging of living biological systems, provide contrast between the various techniques, and discuss future directions of development within this area.

2. CLOSED-CHANNEL METHODS

Liquid flow cells (Figure **3**) represent the foundation of the microfluidic closed-channel devices that comprise the majority of devices in use at various synchrotron facilities today. First employed by Wieliczka *et al.* [67] in 1989 to measure the absorption coefficients of water, their basic structure is a micron-scale thick gasket pressed between two infrared crystals and mechanically assembled as a stack in an external manifold that may also allow for sample injection and temperature control. This configuration is generally used for transmission experiments and allows for a sample to be maintained in relatively uniform, thin layer of water with absorption signal below saturation so that the water background can be subtracted later to obtain the sample spectra [66, 68-70]. This scheme is versatile in that a variety of different windows may be used and it is typically demountable, so that flow cells can be disassembled for cleaning and reuse. The main drawback of demountable flow cells is that the

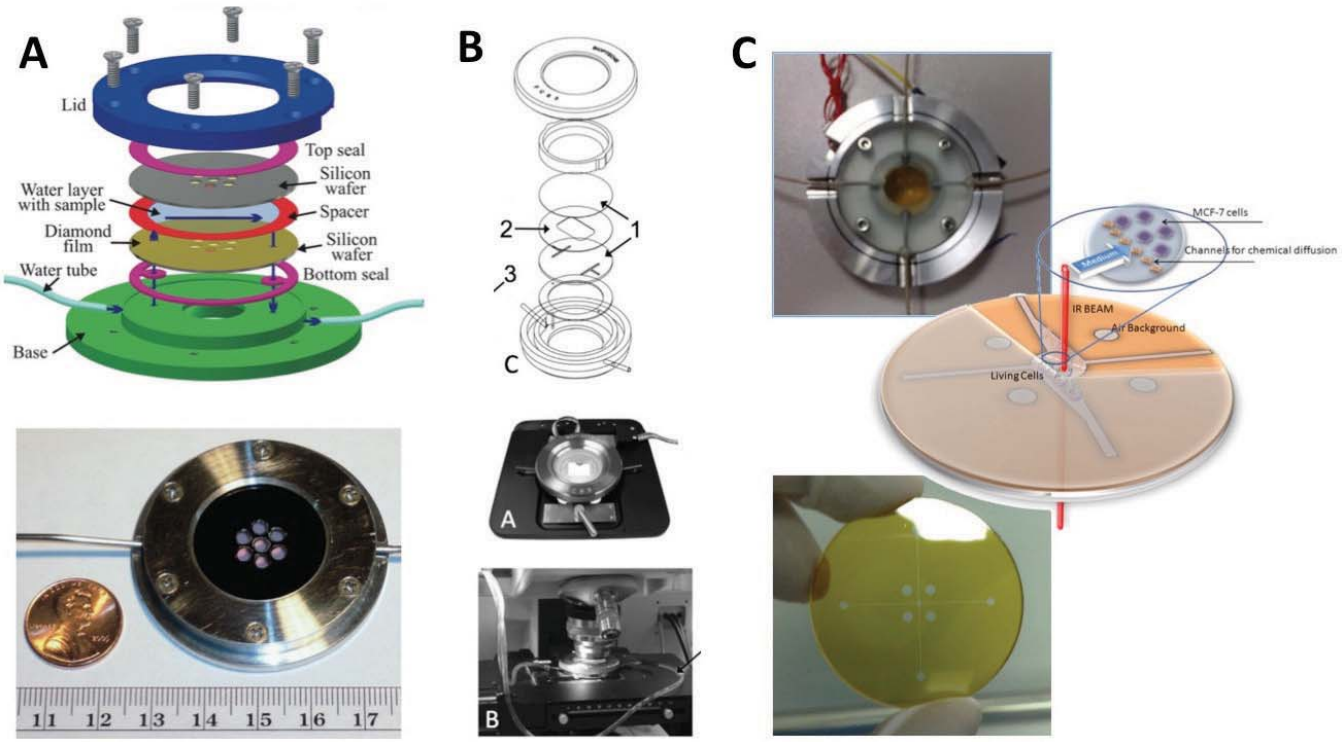

Figure 3. Closed-channel devices – **A**) Demountable stack-assembled flow cell (adapted from Nasse *et al.* [74]) to measure biological specimens with high spatial resolution. Cells are visualized through diamond films grown on silicon wafer and seals are maintained by mechanical pressure from assembled manifold. The flow cell can be disassembled after measurement to reuse parts or access cells. **B**) Demountable stack-assembled flow cell (adapted from Tobin *et al.* [73]) with machined features on separate layers. Based on a modified Bioptechs FCS3 cell, the channel and chamber layers are pressed between two CaF_2 crystals for transmission measurement on a Bruker Hyperion microscope. **C**) Microfabrication allows more complex structures to be integrated into the flow cell and very precise control of water film thickness to be maintained uniformly over the entire measurement area. Device shown from Grenci *et al.* [87] has channels defined in a photosensitive polymer that covers the CaF_2 window. The device is permanently sealed by thermomechanical bonding.

path length, which relies on mechanical pressure, is not easily reproducible between measurements, increasing bias when comparing data from different experiments. Poor sealing, leakage, and limited experimental complexity are two additional shortcomings when it is compared to approaches that use microfabrication.

In 2005, Moss *et al.* [71] were the first to apply flow cell techniques to SR-FTIR imaging of single living cells. Human colorectal cancer cell lines (HT29, SW-480, WIDR, $CaCO_2$), human fibroblasts (primary culture) and human umbilical vein endothelial cells from healthy controls, in both confluent and exponential cultures were plated onto one CaF_2 window. The window was mounted in a standard liquid cell (SpectraTech EZ-Fill) with a 15 µm Teflon spacer and closed with another window to limit the water film thickness. Around the same time, Miljkovic *et al.* [72] also reported imaging live HeLa cells suspended in growth medium, acquired in both reflection and transmission modes, on different substrates (low-e slides and CaF_2) and with spacers of different thicknesses using benchtop FTIR equipment. Heraud *et al.* [42] then built an in-house liquid cell based on the SpectraTech cell and demonstrated SR-FTIR mapping of the metabolite distribution in living algae *Micrasterias hardyi*. These proofs-of-principle experiments demonstrated the viability of microfluidic devices for SR-FTIR measurement of living cells.

The work that followed received significant interest from the IR community, particularly the IR synchrotron facilities [73-78]. Closed-channel configurations became a focal point and evolved into two main branches: One with demountable flow cell using plastic spacers and another micro-fabricated flow cells (both demountable and permanently sealed).

Demountable, stack-assembled cells inherited the basic layout of flow cells but added versatility for window selection and high-resolution imaging. The most common window material is CaF_2 since it has good IR transparency, is relatively insoluble in water, and is cheap and robust compared to other materials such as BaF_2 or ZnSe. In order to use high magnification objectives with small working distance, Nasse *et al.* [74] developed a demountable liquid cell with the viewports made of 0.4-0.8 µm diamond films grown by chemical vapor deposition on a silicon wafer. The total device thickness including the holder was only a few millimeters to take advantage of the high resolution imaging capabilities at the IRENI beamline and conform to the spatial constraints therein. Stack-assembled devices have been used to measure protein expression in live cells [79,80], DNA conformational changes [81], and monitor progression of the cell cycle [82]. This technique does not require microfabrication, and can mostly be implemented with off-the-shelf components outside of the custom manifold and machining through-holes in one of the windows. The drawback of this

approach is the difficulty to implement sophisticated structures within the spacer and the path length reproducibility issues related to the mechanical clamping of the stack, giving variable compression of the spacer.

To cope with some of the limitations of stack-assembled cells, microfabrication has emerged as a method to implement more complex spacer designs and to build completely sealed, FTIR-compatible devices for live cell measurement. Hinsmann [83] performed the first use of microfluidic devices with infrared spectroscopy in 2001. With a mixer microstructure made between two calcium fluoride crystals, they were able to observe the real-time saponification of methyl monochloroacetate with sodium hydroxide. This approach was translated to living biological system imaging in 2010 by Birarda *et al.* [75] and Tobin *et al.* [73], where spacers were directly fabricated on top of IR-transparent windows using optical lithography or direct printing methods [84]. This method allowed microstructures like channels and reservoirs to be added to the cell chamber [85,86] or to change the material surface properties [87,88] while giving more precise control of the spacer thickness. The fabrication approach further allowed development of devices that were either thermo-mechanically or chemically sealed [59, 63] instead of being clamped together.

A few examples of the application of microfabricated devices include assessment of the effect of different fixatives and fixation protocols on living human monocytes [59], identification of the spectral markers of apoptotic and pre-apoptotic cells [89], monitoring the progression of cell cycle [90], and evaluating the chemical response of leukemia cells to drug treatments [91].

Some concerns have been raised with closed-channel devices in terms of the effects of both confinement and contact with non-conventional materials on the viability of cells over extended measurement periods. The use of deuterated water D_2O [43,92] has been attempted to loosen the spatial constraint in the liquid film thickness to about 20 μm. However, it introduced other problems because cell viability cannot be sustained for long periods in D_2O and isotopic exchange caused difficulties in water compensation during analysis. Birarda *et al.* [93] evaluated the effect of confinement on a circulating cell line. Monocyte cells (U937) were confined in devices having different thicknesses ranging from 9 (i.e. similar size of the cells) to 3 micrometers. Observation of the time evolution of DNA, protein and lipid bands showed that no cellular response was detectable for deformations lower than 60% for 100 minutes upon entering the device. This is an important consideration when working with large cells that may experience significant deformation to fit within 9-10 μm thick devices necessary for measurement.

Deformation is less of a concern for adherent cells but their interaction with the surface of infrared transparent materials may cause cytotoxicity problems. Wehbe *et al.* [94] found the infrared transparent Si, ZnS, and CaF_2 did not impact the viability of adherent mammalian cell lines, but BaF_2 and ZnSe did Mitri *et al.* [88] then demonstrated a nanometric layer of silicon dioxide can be used to protect cells from BaF_2 and enable its exploitation of better transparency at lower frequencies. It remains an issue that prolonged exposure to shear stress during perfusion in closed-channel devices can damage cell viability [95] and has a limited measurement period of approximately 48 hours [87].

3. OPEN-CHANNEL METHODS

A relatively new approach in microfluidic devices for live cell imaging is the application of open-channel devices (Figure **4**). These are characterized by having one surface of the fluid exposed to atmosphere and the liquid thickness controlled by surface effects. In 2009, Holman *et al.* [96] used continuous flow of fluid through a microchannel etched in silicon, supplied by a combination of hydrostatic pressure and capillary forces. Hydrophobic treatment to other surfaces ensures water flows exclusively within the channel and forces were carefully balanced between the hydrostatic pressure of a feeder droplet, and capillary pull into a cleanroom tissue to maintain constant fluid thickness. This method was used to track the development and growth of an *E. Coli* biofilm over the course of two days using transmission measurement.

Loutherback *et al.* [97] recently demonstrated a second approach using an IR-reflective porous membrane to measure adherent mammalian cells for up to a week. Mammalian cells plated on top of the membrane were maintained in a thin layer of fluid above a larger flow channel from which they can draw fluid by capillary action. A thin (15 nm) gold layer deposited on top of the membrane allows FTIR measurement to be performed in a transflection mode. This device has allowed continuous measurements of live cells for up to seven days. The primary chemical observation showed an increase in carbohydrates associated with surfactants to manage evaporative stress by the cells, which suggests that a porous membrane-based approach is best suited for cells that naturally grow at air-liquid interfaces such as epithelial tissues of the skin, lung, eyes or microbial biofilms.

The advantage of the open-channel approach is that the fluid thickness can be maintained much thinner than that allowable by closed-channel devices, potentially reducing or removing the need to perform water background subtraction. These devices also reduce or avoid entirely the fringing effect from interference of multiple reflections at the infrared window surfaces by allowing the upper window to be placed much further from the lower window. A disadvantage of this approach is that environmental conditions must be maintained at high humidity to ensure that evaporation does not remove the media covering cell surface and the biological system must be chosen carefully to suit the environmental conditions.

4. FUTURE DIRECTIONS

Figure **5** highlights future directions. Closed-channel devices using microfabrication can gain significant additional features with the incorporation of various well-developed microfluidic modalities [98]. The incorporation of cell traps [99,100] may be beneficial to place cells in well-registered locations for time-course measurements. Such structures may also help hold motile cells in place during measurement [43]. Water-in-oil droplets further may provide an alternative opportunity to encapsulate single cells [101]

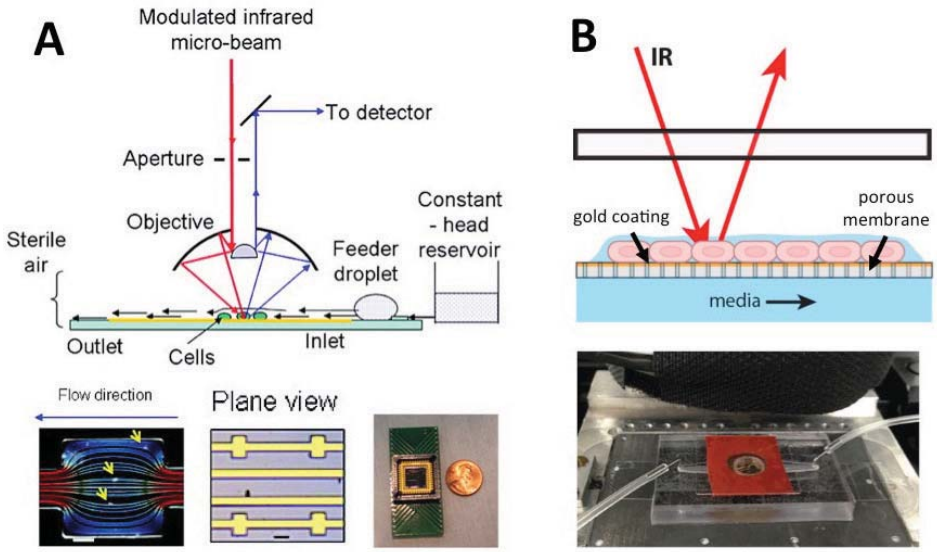

Figure 4. Open-channel devices – **A**) Open-channel device to study live bacteria in aqueous environments (adapted from *Holman et al.* [96]). The device is composed of channels etched into a silicon wafer. Flow of media is maintained in channels by hydrophobic treatment to non-channel surfaces and liquid flow is controlled by hydrostatic pressure in a feeder droplet. **B**) Membrane device to study cells that grow at the liquid air interface (adapted from Loutherback *et al.* [97]). The cells are maintained in a thin layer of water on top of a gold-coated porous membrane. Constant flow of media underneath the membrane allows for nutrients to be replenished by capillary action.

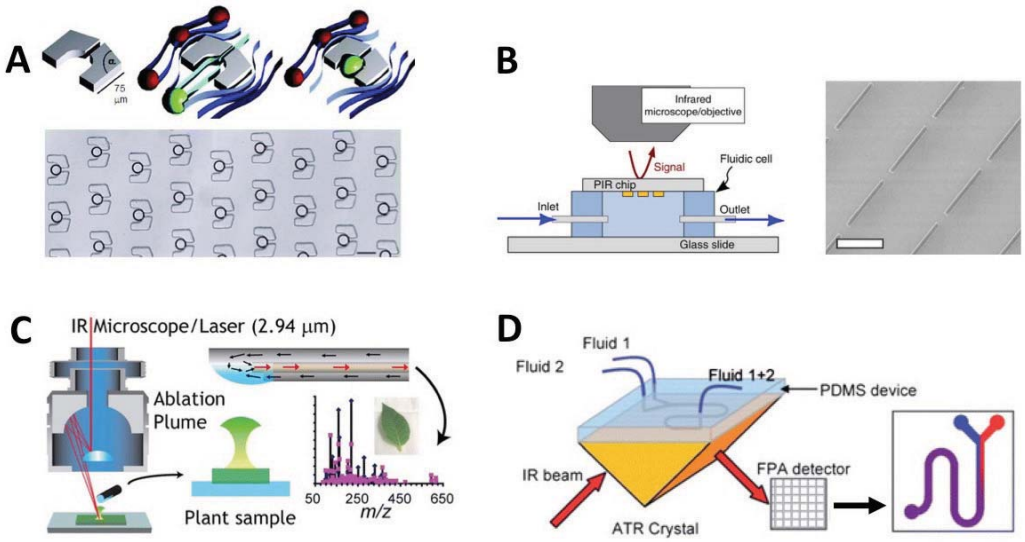

Figure 5. Future directions in microfluidic FTIR devices - A) Advanced microfluidic structures such as droplet generators and cell traps could be used for sample confinement and entrapment in an array format for measurement with SR-FTIR (adapted from Huebner *et al.*). **B**) Plasmonic microstructures can be used to increase the sensitivity of IR spectromicroscopy to extremely dilute analytes in solution (adapted from Adato *et al.* [112]). **C**) SR-FTIR in open-channel devices can be hyphenated with mass spectrometry for more detailed molecular identification as demonstrated by O'Brien *et al*. [57]. **D**) The coupling of SR illumination with large area focal plane array (FPA) imaging detectors can be used to employ SR for ATR imaging and fluidic micro incubators can be used for experiments on live cells (adapted from Chan *et al.* [108]).

and monitor metabolic activity in well-characterized and isolated environments [102].

One development that may prove particularly valuable is the ability to deposit smooth, IR-reflective layers of gold on polydimethylsiloxane (PDMS) [103]. While there remains concerns about electric field standing wave effects during transflection measurement [104], this material would allow cheap, rapid device fabrication and the inclusion of multi-layer structures with a large suite of features including valves and mixers [105]. Mating through-holes in closed-channel devices to a PDMS-based, multiplexed in/out manifold may provide similar benefits while still allowing transmission measurements [106].

In addition to the open- and closed-channel devices, a third group of devices that are prominent in conventional FTIR imaging but have yet to see much use with synchrotron

illumination sources are attenuated total reflection (ATR) cells or chambers. These ATR-based devices are functionally closer to environmental chambers than flow cells or microfluidic devices. ATR probes only a few microns at best at the crystal-liquid/cell interface, so the thickness of the total layer of water is not important, and it is possible to achieve petri dish-like condition for cell growth [107-111].

Another exciting area of future development is the integration of live-cell imaging with plasmonic nanoantennas for ultra-sensitive surface enhanced IR absorption spectroscopy. This could enable trace detection of physiologically significant yet elusive molecular species such as metabolites, cytokines, growth factors and antibodies. Towards this end, Adato *et al.* [112] have demonstrated an ultra-sensitive plasmonic internal reflection chip able to monitor monolayer protein binding events in real time with 10-fold signal enhancement over ATR.

Multimodal imaging in combination with mass spectrometry is also a promising area of future development. Infrared spectral data lacks sufficient chemical specificity for unique molecular identification. High-resolution mass spectrometry (MS) can make possible more complete identification of the full range of molecules involved in functional metabolism, including elemental composition obtained by accurate mass measurements and structural information gained from fragmentation products formed in tandem mass spectrometry measurements [113]. Combining these two techniques, using a demountable flow cell or open-channel device of certain kind, will allow the non-destructive, ambient chemical monitoring capabilities of SIR spectromicroscopy to be paired with efficient, spatially-resolved analysis by mass spectrometry.

5. CONCLUSION

Synchrotron-radiation Fourier transform infrared spectral microscopy has progressed rapidly in the last decade. Real-time measurements of biological processes in living cells have been and will continue to be one of the most exciting developments within this area. Various microfluidic devices have emerged as a platform for meeting the challenging requirement of providing both a hospitable environment where cellular processes and responses can be probed over many hours or days while maintaining water films thin enough to obtain high quality spectral information. Although it is in its infancy, and more work is needed before its full potential can be realized. The foundations of the field are already quite strong. The device development in this area is expected to proceed to improve environmental control and enable additional capabilities such as sequential chemical treatments, advanced microfluidic structures, and multimodal imaging. Other paradigms such as ATR or plasmonic antennas may allow circumvention of water thickness requirements and open whole new areas of application. Across all device designs, careful thought must be put into experimental preconsiderations for the biological system of interest, as each design has distinct strengths and weaknesses.

From infrared absorption data, spatially- and temporally-resolved chemical information, including the distributions and relative abundances of the classes of chemicals such as proteins, lipids, carbohydrates, or metabolites, is obtained. However, infrared data lack sufficient molecular or chemical specificity for unique identifications. The advent of genetically encoded labels enables the non-destructive fluorescence microscopy techniques [114,115] to provide highly selective and specific spatio-temporal information on the targeted cellular components or signalling molecules. Meanwhile, the destructive but high-chemical-resolution mass spectrometry (MS) can make possible more complete identification of the full range of molecules involved in functional metabolism, including elemental composition obtained by accurate mass measurements and structural information gained from fragmentation products formed in tandem mass spectrometry measurements [116-119]. Together, they will offer great, perhaps even revolutionary new capabilities for the future of SR-FTIR imaging of living biosystems.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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