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Development of single-beam wide-field infrared imaging to study sub-cellular neuron biochemistry☆

Mark J. Hackett [a,*], Sally Caine [b], Xia Liu [c], Tim E. May [c], Ferenc Borondics [c]

[a] Molecular and Environmental Sciences Group, Geological Sciences, University of Saskatchewan, 114 Science Place, Saskatoon, SK S7N5E2, Canada
[b] Department of Anatomy and Cell Biology, University of Saskatchewan, 107 Wiggins Rd., Saskatoon, SK S7N5E5, Canada
[c] Canadian Light Source, SK, Canada

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ABSTRACT

Multi-beam wide-field imaging using synchrotron mid-infrared light sources coupled with focal plane array detectors has provided a major breakthrough to the field of bio-spectroscopic imaging. The ability to collect sub-cellular molecular images in minutes has opened the door to a new era of biochemical studies. Although a multi-beam approach is the superior method to this form of imaging, it requires a specialized set of beamline optics, which may not be compatible with existing mid-infrared microscopy beamlines, or research programs/applications currently in place (some of which do not require an imaging component). In this investigation we demonstrate that a single-beam approach can be utilized in a similar manner to multi-beam imaging, to collect sub-cellular biochemical images of brain neurons in a rapid time frame, without extensive modification of an existing beamline configuration. This study uses an applied example, imaging the same neuron *in situ* within a brain tissue section, with both synchrotron and thermal sources. The results highlight the advantage of improved spatial resolution/image quality and spectral quality (signal to noise ratio) that is obtained when a high magnification and high numerical aperture objective (52×, 0.65) is coupled to a synchrotron mid-infrared lightsource with a focal plane array detector. The approach we report may prove to be particularly appealing to numerous existing mid-infrared beamlines, allowing straightforward integration of sub-cellular biochemical imaging with existing non-imaging research applications.

1. Introduction

The spatial resolution that can be achieved for imaging techniques utilizing far-field detection is limited by diffraction. Due to the long wavelengths of light across the mid-infrared (mid-IR) range, the best possible spatial resolution that can be achieved with infrared spectroscopic mapping/imaging is typically 2–10 μm [1]. Although this is approximately an order of magnitude poorer than the spatial resolution that can be achieved with conventional light microscopy in the ultra-violet or visible light range, it is still sufficient to study biological samples at the single cell, or sub-cellular level [1,2]. Typically to achieve diffraction limited spatial resolution across the mid-IR range, the high photon flux from a synchrotron light source that enables the use of small apertures in a confocal microscopy arrangement is required. This method of data collection, where the sample is step scanned ("mapped") through a small focused beam with a confocal microscopy configuration is termed synchrotron radiation-FTIR-microscopy (SR-FTIRM) [1–4]. However, due to the "step by step" mode of data collection, even with a synchrotron light source and reduced data collection time compared to a thermal source, data acquisition times may verge on impractical for biological studies (*i.e.*, many hours to image a single sample) [4]. In addition, the use of small apertures necessitates the use of an even smaller step size when mapping a sample to achieve a sufficient oversampling factor to provide maximum image contrast, which further extends the acquisition time [5]. Moreover, if diffraction limited spatial resolution is to be achieved for bands at shorter wavelengths, a significant decrease in the signal to noise ratio SNR will be present across the spectral range of longer wavelengths [6]. Likewise, if diffraction limited resolution with suitable SNR is desired at longer wavelengths, then bands at shorter wavelengths will not be mapped at the diffraction limit [6]. Therefore, although diffraction

Abbreviations: FPA, focal plane array; FTIRI, Fourier transform infrared imaging; FTIRM, Fourier transform infrared microscopy; SR, synchrotron radiation; N.A., numerical aperture.

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* Corresponding author. Tel.: +1 306 260 2773.
E-mail address: mark.hackett@usask.ca (M.J. Hackett).

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limited spatial resolution can be achieved with SR-FTIRM, the practical constraint of time and the broad wavelength spread in the mid-IR region prevent many studies attaining this limit. Nonetheless, coupling FTIR spectroscopy with microscopy and synchrotron radiation light sources has provided enormous advances to the field of bio-spectroscopic imaging [7–14].

Secondary to coupling FTIR spectroscopy with microscopy and synchrotron light sources, a major revolution in infrared detectors has occurred. Namely, the widespread use of focal plane array (FPA) detectors has provided the ability to simultaneously collect several thousand spectra (*i.e.*, 4096 spectra for a 64×64 pixel array) over a relatively large area. FPA detectors were relatively easily adapted to laboratory based thermal sources, and almost instantaneously paved the way for rapid, high throughput FTIR-imaging (FTIRI) [15]. Indeed, thermal sources in laboratory based instruments provide homogenous illumination of FPA array detectors with sufficient SNR when using a 15× magnification objective (N.A. 0.4) and are well suited to rapidly image larger tissue areas [3,15–17]. However, they do not provide sufficient brightness to allow the use of higher magnification and higher numerical aperture objectives (*i.e.*, 52× or 74× and 0.65 N.A.). Therefore, laboratory based instruments with a thermal source do not provide diffraction limited spatial resolution imaging with sufficient spectral SNR within a realistic experimental time frame. Following the success met with coupling FTIR spectroscopy with thermal sources and FPA detectors, coupling synchrotron light sources with FPA detectors was a logical step for the bio-spectroscopic imaging field. This was achieved by several research groups, with the goal of diffraction limited imaging and a large oversampling factor, in a short time frame [2,4,18–29]. It was hypothesized that the high brightness of a synchrotron light source would enable the use of high magnification and high numerical aperture objectives. In accordance with Eq. (1), the use of high numerical apertures reduces the theoretical spatial resolution (d) that can be achieved. The use of high magnification objectives provides a substantial oversample factor, provides greater chemical contrast in the images, and allows the theoretical spatial resolution to be achieved. In addition, the use of FPA detectors would allow many spectra to be collected simultaneously, significantly reducing data collection time. Unfortunately, serious limitations with this approach were encountered due to the small source size that does not homogenously illuminate an entire FPA detector.

$$d = \frac{0.61 \times \lambda}{\text{N.A.}} \quad (1)$$

Specialized optical designs were developed to overcome these limitations, which incorporated multiple beams to illuminate the entire focal plane array. This form of multi-beam wide-field synchrotron radiation FTIRI (SR-FTIRI) was implemented at a dedicated beamline at the synchrotron radiation center, Wisconsin (IRENI beamline), and incorporated 12 beams, illuminating an array area up to 96×96 pixels in size through a 74× objective by defocusing the condenser objective of the microscope to homogenize the field [21,22,25–27]. A simplified approach, modified an existing mid-IR microscopy beamline, and illuminated a 32×32 pixel area of an FPA through a 74× objective, with 4 defocused beams [24]. Both cases resulted in an effective pixel size of $0.54\,\mu\text{m} \times 0.54\,\mu\text{m}$ and rapid biochemical imaging at diffraction limited spatial resolution was achieved, highlighting the tremendous potential of the method.

An unfortunate limitation to multi-beam wide-field SR-FTIRI is that a dedicated beamline or dedicated optical configuration is required. This excludes the use of this method at many existing mid-IR beamlines, and also prevents rapid interchange between bio-spectroscopic imaging studies and other uses of mid-IR light at synchrotron sources. Therefore, in this investigation we have examined the capabilities of performing single-beam wide-field SR-FTIRI at a synchrotron light-source, Canadian Light Source (CLS), without extensive modification to existing beamline optics. A single-beam approach allows rapid interchange between imaging and non-imaging uses of a synchrotron mid-IR microscopy beamline, and could be implemented at any existing beamline. As demonstrated by others, the foot-print of a defocused synchrotron beam can be as large as $\sim 30\,\mu\text{m} \times 30\,\mu\text{m}$ (a defocused beam of $20\,\mu\text{m} \times 20\,\mu\text{m}$ is used in this study), which is slightly larger than a typical cell ($\sim 15\,\mu\text{m} \times 15\,\mu\text{m}$) [30]. This study demonstrates that coupling a synchrotron light source, with a high magnification and high numerical aperture objective (52× N.A. 0.65), and an FPA detector, can achieve *in situ* sub-cellular biochemical imaging of rat cortical pyramidal neurons. This approach does not yield the same oversampling factor that can be achieved with higher magnification objectives used in other studies (*i.e.*, $0.54\,\mu\text{m} \times 0.54\,\mu\text{m}$ with a 74× objective, $0.77\,\mu\text{m} \times 0.77\,\mu\text{m}$ with a 52× objective), and the sampling area is smaller ($20\,\mu\text{m} \times 20\,\mu\text{m}$). Nonetheless, the use of a 52× objective provides superior image resolution and chemical contrast compared to a benchtop instrument and superior SNR is achieved with a synchrotron light source compared to that of a thermal source.

A specific research area where the ability to resolve sub-cellular biochemistry would be of great benefit is the field of neuroscience. Many bulk biochemical alterations are known to occur during numerous brain diseases. However, a complex and heterogeneous organization of many different types of cells exists in brain tissue (neurons, inter-neurons, and glia), and numerous connections and chemical cross talk occur between them. Therefore, co-localization of a wide range of biochemical parameters to individual brain cells is impossible through biochemical assay, and difficult with conventional light microscopy due to the limited amount of chemical information obtained through cytochemical methods. The ability of multi-beam wide-field SR-FTIRI imaging to study neuronal biochemistry at the sub-cellular level has recently been reported [31–33], and has provided an unprecedented level of neuro-chemical insight at the micron level. This study demonstrates that single-beam wide-field SR-FTIRI can be used to study the sub-cellular biochemistry of brain neurons *in situ*, without extensive modification to an existing mid-IR microscopy beamline, an exciting prospect for future neuroscience research at synchrotron light sources. We note that although brain neurons have been investigated in this study, this imaging method would be widely applicable to any biological sample or imaging investigation in general.

2. Materials and methods

2.1. Animal models

Brain tissue was obtained from a healthy 6 week old male Sprague-Dawley rat used in a previous study, where the rats were housed with a 12 h light/12 h dark cycle with ad libitum access to chow and water [31]. This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

2.2. Sample preparation

To avoid introduction of chemical artifacts that can result during sample preparation of biological samples [34], the rat was anaesthetised with isoflurane, and humanely sacrificed through decapitation, with the head immediately frozen by dropping into