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Biomolecular imaging of host-pathogen
interaction by Raman micro-spectroscopy

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ABSTRACT

The ability of pathogens and their surrogate host cells to exchange molecules is at the core of all host-pathogen interaction processes. Small chemical molecule signals provide a rich vocabulary for cellular communication, and pathogens have evolved the capacity to modulate, mimic or hijack these signals in a defensive, exponentially increasing the scope and complexity of this chemical language between host cells and pathogens. The objective of this work is to use Raman microscopy (RMS) to analyze molecular changes during infection of human cells by intra- (*Toxoplasma gondii*) and extra-cellular (*Acanthamoeba castellanii*) protozoan parasites. The work is divided into two parts. Firstly, we studied host-pathogen biomolecular interaction by using RMS. Secondly, we employed stable isotopes substitution technique to induce spectral changes that are specific to the labelled molecules. In this approach mammalian cells are selectively grown in culture medium in which only certain molecules are substituted with stable-isotope counterparts, and the exchange of these molecules are monitored between individual mammalian cells and parasites in real-time. Our results show that Raman spectroscopy can be a great tool to understand the molecular processes that mediate the interaction between the pathogens and host cell by providing quantitative spatially- and temporally-resolved information regarding molecular trafficking and exchanges. A better understanding of these complex interactions will advance our understanding of microbial pathogenesis and potentially identify new therapeutic targets.

List of Publications and Conferences contributions

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Dedicated to my Parents

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Chapter 1

Introduction

This chapter provides the motivation of the research presented in the thesis. It gives an introduction to the parasites studied and diseases caused by these parasites, and why Raman microspectroscopy is used for the measurements of host-pathogen interaction. The study is based on the two types of parasites that were used as models for intracellular and extracellular parasites as described in the next sections

Toxoplasma gondii (*T. gondii*) is emerging as a model system to study the mechanisms of intracellular parasites infection biology as it can infect any nucleated cells of any vertebrate species [1]. *T. gondii* is one of the most important obligate intracellular apicomplexan protozoan pathogens affecting the central nervous system and causing serious neurological disease in humans and animals [2]. Even though most *T. gondii* infections are benign, this pathogen can cause significant morbidity during pregnancy and in immuno-suppressed patients including brain and visual impairment [3]. In humans, *T. gondii* is most frequently involved in the causation of infectious uveitis [3]. In dogs and cats, *T. gondii* is a significant cause of ocular morbidity including inflammation and scarring of the retina (retinitis), iris (uveitis) and cornea (keratitis), resulting in

severe pain and welfare implications. The aim of the study is to understand the molecular changes that occur within the host cell when parasites enter and multiply within these cells. The mechanism of infection of *T. gondii* is not fully understood yet at the molecular level [4].

The model used for extracellular parasites is *Acanthamoeba castellanii* (*A. castellanii*). *Acanthamoeba* species, discovered in 1930, are the most prevalent free-living protozoa and inhabit a variety of environments including air, soil and water [5], swimming pools [6], eye wash stations [7], and noses and throats of asymptomatic individuals [8]. *Acanthamoeba* can cause *Acanthamoeba* keratitis (AK) and granulomatous amoebic encephalitis (GAE), two severe, hard to treat and potentially fatal diseases [9]. AK is an infection of the cornea causing severe inflammation, intense pain, impaired vision, and blindness if left untreated [10, 11]. Corneal trauma and subsequent exposure to *Acanthamoeba* or transfer of *Acanthamoeba* to a damaged corneal surface by improperly maintained contact lenses can both result in this progressive sight-threatening corneal disease. Diseases caused by *A. castellanii* infection are not well understood despite their debilitating and life-threatening consequences due to various experimental and conceptual challenges. Some pathogenic mechanisms have been partially characterized, however in many cases better description is yet to be achieved.

In order to better understand the pathogenesis of the parasite it is critical to identify the molecules that mediate the interaction between this parasite and the host cells. However, the host–pathogen interaction involves highly complex time-dependent molecular interactions. Extracting quantitative information

without disturbing the cell integrity, while preserving molecular specificity and high spatial resolution remains a grand challenge when imaging individual living cells. Microscopy techniques have contributed significantly to the understanding biology of these parasites [12]. However, although currently available conventional imaging techniques provided a considerable amount of information, they are still insufficient to provide an improved understanding of the mechanism at molecular level. Most conventional imaging techniques used in biomedical sciences require cell labelling with fluorescent dyes in order to access specific molecules. They may also require cell fixation, lysis [13] or other immune-staining methods based on the detection of particular antibodies [14-25]. As these procedures are invasive for the cells, and have limited stability, there is a need for non-invasive technique to obtain information about the properties of the cells. Label-free microscopic techniques such as phase-contrast and dark field give only morphological information about the cell and cannot provide chemical information.

Recent non-invasive molecular imaging techniques using vibrational spectroscopy, such as Raman micro-spectroscopy (RMS), have contributed in various fields of life sciences. RMS is a well-established analytical technique that enables chemical analysis of individual cells and bacteria with submicrometric spatial resolution [26-30]. RMS was also used for time course experiments on individual live cells revealing molecular processes not attainable with other imaging methods [31-35].

The metabolic alteration in both parasite and host cell is an area which requires further investigation, for example parasite's access to host nutrients in infected tissues, which is fundamental for the parasite growth and disease progression. Our understanding of this crucial process is still limited because of various technical and conceptual challenges. This work is based on single cell Raman spectroscopy imaging to study the interaction of protozoan parasites with human host cells. Raman Spectroscopy can be even enhanced to trace the specific molecules by using stable isotope labelling of the aromatic amino-acids (AA), such as phenylalanine (Phe), which has been used and combined with Raman microspectroscopy (RMS) in the present work. This integrated approach has enabled real-time detection of AA exchange during the interaction of the apicomplexan protozoan parasite *T. gondii* with the human retinal pigment epithelial cells (ARPE-19). Also, it allowed real-time tracking of the labelled Phe non-invasively on a single cell level and with micrometer spatial resolution. The same approach is used to study the uptake of the deuterated Phenylalanine (L-Phe(D8)) by extracellular *A. castellanii* trophozoites from ARPE-19 host cells.

CHAPTER 2 introduces the basic cell and parasite biology concepts required in this thesis, techniques used in cell study and Raman spectroscopy for cell and pathogen study. This chapter also explains the basic theory of Raman Spectroscopy.

CHAPTER 3 describes the instrument's details. It explains the methods for spectral calibration and the spatial resolution of the Raman micro-spectrometer

system. Also, this chapter gives brief overview of the fluorescence imaging system.

CHAPTER 4 describes the experimental results of investigating the interaction between the apicomplexan protozoan *T. gondii* and host cells using label-free Raman Spectroscopy.

CHAPTER 5 presents experiments performed on tracing amino acid exchange during host-pathogen interaction by combined stable-isotope time-resolved Raman spectral imaging.

CHAPTER 6 presents the study of tracking amino acid uptake into the protozoan *A. castellanii* by stable-isotope labelling and Raman spectral imaging.

CHAPTER 7 discusses the major findings, and suggests future areas of research.

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Chapter 2

Background

A parasite is an organism that lives on another organism known as the host. Parasites develop a relationship with the host in order to get all the nutrients, ultimately harming and possibly causing the death of the host. The parasite lives on or in the body of the host. This chapter introduces the basic concepts in cell and parasite biology and techniques used for their study. This chapter also includes a brief description of Raman scattering.

2.1 Basics of cell biology

The cell is the structural and functional unit of all living organisms. There are many types of cells according to their functions in the organism. Some of the major features that characterize the typical cell are the same in nearly all cells [1]. Cells interact with their environment to uptake nutrients and these chemicals can be converted into a wide variety of other chemicals to obtain energy to support the numerous cellular processes or to synthesize larger cellular components. A cell can synthesize its own components, and repair the worn-out parts [1]. The cells can grow and reproduce. A cell enlarges then divides to create two cells which can further grow and reproduce [1].

The structure of a typical eukaryotic cell is shown in Figure 2.1. The cell is enclosed in a cell membrane made up of a phospholipid bilayer, which separates the cell interior from its external environment. The membrane acts as a semi-

permeable barrier, allowing the controlled entry and exit of the chemicals into and from the cells. Most of the cell volume is occupied by the cytoplasm in which all cell organelles can be found. The main component of cell is the cell nucleus, where the DNA (deoxyribonucleic acid) is present. The DNA carries the genetic information of the cell. The nucleus also has a nuclear membrane around it, and nuclear pores within the membrane allow the transport of molecules between cytoplasm and nucleus [1]. Other important components of the cell include the endoplasmic reticulum, mitochondria, Golgi apparatus, ribosomes and cytoskeleton. The endoplasmic reticulum is important for protein synthesis, ribosomes are the sites where RNA (ribonucleic acid) is translated into proteins. The cytoskeleton is formed of actin and other protein filaments and is responsible for the shape of the cell [2, 3].

The mitochondria are very important cell components known as the “power house” of the cell because they generate most of the energy required for the cell to function. The mitochondria are 0.5-1.0 μm in diameter and 1-3.0 μm in length [4]. They are surrounded by two membranes, an outer membrane and an inner membrane, which is arranged into complex infoldings called cristae [1]. The inner membrane is responsible for oxidative phosphorylation, it contains an enzyme that participates in adenosine triphosphate (ATP) synthesis [1]. The number of cristae per mitochondrion tends to be greater for mitochondria with a more intense metabolic activity. The mitochondria are also involved in cellular differentiation, cell death, cell cycle and cell growth [5], as well as in many human diseases, including mitochondrial disorders [6], aging process and cardiac dysfunction [7].

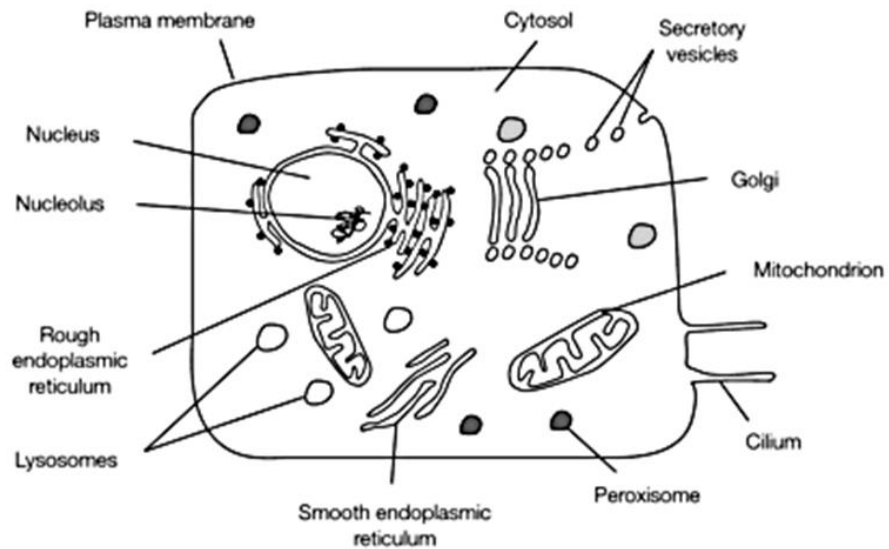


Figure 2.1: A typical Eukaryotic (animal) cell structure [8].

2.2 *Toxoplasma gondii*

Toxoplasmosis is the disease which is caused by the unicellular parasite called *Toxoplasma gondii*., which is a protozoan intracellular obligate parasite belonging to the phylum Apicomplexa [9]. It was first described in 1908 by Nicolle and Manceaux in North Africa and by Splendore working in Brazil [9]. *T. gondii* takes different forms in its life cycle, the oocyst, the trachyzoites and the bradyzoites-containing tissue cyst. Animals of the family Felidae are the only definitive host, replication of the parasites occurs in the intestine of the cat. Oocysts are shed in the faeces of the cat for 7-21days after infection. Infection is acquired by ingestion of food or water contaminated with oocysts from cat faeces. Parasites start replicating inside host cells rapidly and develop to trachyzoites, which are the most active stage of the parasites. They are crescent or oval in shape, 2-4 μ m in diameter and 4-8 μ m in length.

Parasites remain encysted if the immune system of the host is strong, and the parasites are known as bradyzoites; this is the slowly dividing form of *T. gondii*.

Bradyzoites remain inside the cyst for the life of the host [9]. If *T. gondii* detects low/no selection from the immune system of the system, such as in immune-compromised people [10, 11], the parasite reactivate and switch to become tachyzoites and can result in potentially fatal infection.

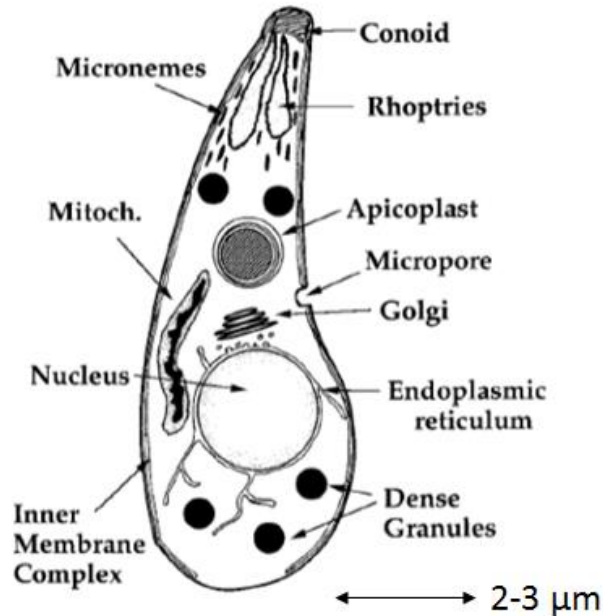


Figure 2.2: The major organelles of the *T. gondii* in the tachyzoite stage [9].

The structure of the *T. gondii* is shown in Figure 2.2 [9]. It contains nucleus, endoplasmic reticulum, mitochondria, Golgi apparatus, some dense granules. The apical end of parasite consists of conoid, micronemes and rhoptries. Rhoptries, micronemes and dense granules play very important part in attachment with the host membrane and are known as secretory organelles [12].

These organelles have their specific protein complement with their time of release according to their role in invasion process [13]. Micronemes are the first to release its secretion to start the invasion process, rhoptries are the second to discharge its content as the invasion progresses and finally the dense granules

release their content when the invasion is essentially complete [13]. The cytoskeleton gives its shape to glide on the surface of the host [14]. The parasites need an environment where they can replicate with no resistance from the immune system on host. To make this happen, parasites influence the host immune cell processes to reduce its effects on parasite proliferation [15]. They also promote anti-apoptotic behaviour inside the host cell [16] for the survival of the host cell to replicate inside it. The parasitophorous vacuole (PV) encapsulates the parasites and protect them from the endolysosomal system responses of the host cell [17, 18]. As they find the way inside the host cell, they form a PV by forming a membrane around them [17, 18]. They keep replicating inside PV with a reproduction time of 6 to 8 hours (*in vitro*) until it ruptures and the parasites are released to infect other neighbouring cells [19].

Infections by *T. gondii* are widely prevalent in all warm blooded animals including humans. It can cause mild flu like symptoms or can be asymptomatic in the few weeks of infection. It can be fatal in immune-compromised people, pregnant women and AIDS patients. If a pregnant woman acquires infection it results in a foetus with neural disorders or spontaneous abortion.

2.3 *Acanthamoeba castellanii*

Acanthamoeba is a genus of amoebae, one of the most common free living protozoa found in soil, and frequently found in fresh water, mammalian cell cultures, vegetables, contact lenses etc. It has two stages in its life cycle namely trophozoites and cysts.

Trophozoite is the active phase of the life cycle, has a diameter of 10-25 μ m [20] and its shape keeps changing due to motion of the parasites, oval to triangular with the help of pseudopods and surface acanthopodia. The cells possess key organelles, including a nucleus, Golgi complex, digestive vacuoles, endoplasmic reticulum, mitochondria (for generating energy required for metabolic activities) and contractile vacuole (for osmoregulation) within a plasma membrane [21, 22]. The acanthopodia play an important role in the organism adherence to biological and inert surfaces [21].

The cyst [23, 24] is the inactive stage of the parasites and resistant to the temperature and pH levels effects. *Acanthamoeba* have many species, most of them are free-living bacterivores, but some can cause infections in humans and other animals.

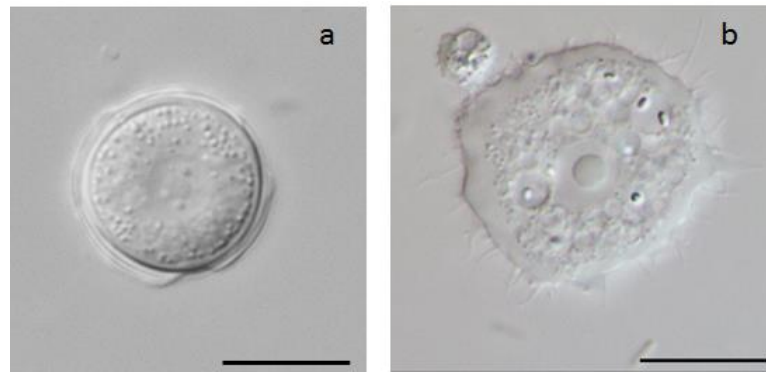


Figure 2.3: *Acanthamoeba* life cycle stages a) Trophozoite b) Cyst. Scale bar: 10 μ m [25].

It enters the body through eyes, nasal passage, and through broken skin. Diseases caused by *Acanthamoeba* include keratitis and granulomatous amoebic encephalitis (GAE) [26] two potentially fatal diseases [22].

Keratitis (AK) is an infection of the cornea. Infection is initiated by trophozoite adhesion to corneal epithelium following which the infection develops as a ring infiltrate. In the latter stages of the disease amoebae invade the stromal layer, with the resulting opacity leading to loss of visual acuity and eventually blindness. Existing corneal damage is known to predispose to the condition, and in addition, more than 80% of AK infections are correlated with the use of contact lenses [27, 28].

In epidemiological terms the infection is comparatively rare. The incidence rate (per 10,000 population) is 0.01 in the USA [30], 0.05 in Holland [31], 0.19 in England [29], 0.33 in Hong Kong [29], and 1.49 in Scotland [30]. However the proportion of the population at increased risk of developing AK may rise as contact lenses continue to gain popularity for ophthalmic and recreational purposes [31, 32].

A. castellanii enters through nasal passage to the respiratory tract, then enters the brain [33] resulting s in brain inflammation and swelling [34] causing death in ~95% of those infected. GAE is often but not always seen in immunosuppressed patients [35].

There are currently no good diagnoses or treatments for *Acanthamoeba* infection. Correct and timely diagnosis, as well as improved treatment methods and understanding of the parasite behaviour are important factors in improving the outcome of infection by this parasite. A recent publication in 2013, has shown substantial effects of some FDA approved drugs with a kill rate above 90% [36] on *in-vitro* studies.

2.4 Methods used to study cells

There are a plethora of techniques developed in the last century to study cells. Some of them are used to measure the morphology of the cells and other are aimed more to look inside the cellular structure and reactions. Since the earliest examination of cellular structures, biologists have been fascinated by observing cells using light microscopy [37]. The advent of fluorescent labeling technologies and a wide range of light microscopy techniques are now available to study dynamic processes in living cells [37].

Light microscopy techniques used to study the morphology of the cell include dark field, phase contrast, evanescent wave, total internal reflectance microscopy (TIRM) and near field scanning microscopy (NSOM) [37]. These are non-invasive techniques and time-lapse imaging of cells is possible. However, these techniques provide only limited biochemical information of the cell systems. Electron microscopy allows imaging of cells with nanometer resolution. Transmission electron microscopy (TEM) has been used to study cell lipid vesicles [38], scanning electron microscope (SEM) is used to study cell cycles over various materials [39]. These techniques are very useful and important in cell structure study at such high resolution scales. But these techniques cannot record time-lapse dynamic events inside cells due to the need for special sample preparations.

Atomic force microscopy (AFM) is used to study the mechanical properties of cell cytoskeleton [40]. This can be used on live cells but has low chemical

specificity. Mass spectroscopy (MS) can be used to characterize the protein, oligosaccharides and nucleic acids which are relatively large molecules with higher molecular weights [41, 42, 43]. Mass spectroscopy is a promising analytical technique in cell study, it identify molecules on the basis of their charge to mass ratio. This is also invasive tool for cell study and cannot be used for live cell measurements.

The biological methods with highest chemical specificity are immunochemistry or immuno-fluorescence, serial analysis of gene expression (SAGE) [44], capillary electrophoresis (CE) [45, 46], western blotting [47] and the polymerase chain reaction (PCR). These methods can give the bio-chemical information about the cell. However, they target only the specific molecules from the whole cell. In addition, these methods are invasive and need laborious protocols to study cells.

Fluorescence microscopy is a well-established biological method, used routinely for cell biology studies. In this method cells are labelled with dye molecules known as fluorophores. The fluorophores are chemically attached to specific molecules inside cell, then the sample is studied by using a wide range of techniques. Confocal fluorescent imaging and multi-photon microscopy have also been used to get the higher resolution images from fluorophore labelled samples [48, 49]. Fluorescent staining methods require cell fixation in most procedures. Fluorescent dyes distribute unevenly throughout the sample and suffers from photo bleaching. Selective staining with fluorescent dye is widely used for fluorescence observation in biological studies, but these techniques give

single time-shots of the cells. Also, the toxicity of most of these dyes excludes their usage for in-vivo studies [50].

Techniques based on the vibrational modes of molecules in cells are chemically specific, non-invasive and provide useful information from complicated systems. Raman spectroscopy is based on the inelastic scattering of light by molecules of the cell. This technique can identify components in cells, tissues and organism based on the vibrational modes of their molecules. Raman spectroscopy is well suited to study cells in aqueous environment, as the water molecules have a small Raman scattering cross-section. Raman measurements are non-invasive, it does not need any sample preparation and extract biochemical information without disturbing the cell integrity. It is possible to repeat the experiment for the same cell and follow the molecular changes during time-dependent biological processes from hours to days.

The first Raman micro-spectrometer was proposed by Dhamelincourt and Delhaye in 1973 [51]. Since then, Raman microscopy was used to acquire the spectra from a tissue sample in 1979 [52] and in 1990 Puppels et al were able to measure the first Raman spectra of living cells [53]. Advances in the past 30 years in instrumentation and data analysis has enabled a broad range of applications from cellular analysis in-vitro to in-vivo clinical imaging [54].

Raman spectroscopy (RMS) has applications in medical, pharmaceutical, forensic, and material sciences. RMS has been used to collect spectra of many drugs, uptake and distribution of drugs, biochemical components of cells [55,53], conformations of protein [56], apoptosis [57-60], differentiation [61]

and for the comparative study between live and dead cells [62]. Raman spectroscopy experiments are able to follow cellular dynamics [63] and continuous biochemical changes throughout the cell cycle [64].

RMS has been used only in few studies to characterize molecular details associated with host– parasite interaction. For example, RMS has been utilized to study the protozoan pathogen *Plasmodium falciparum*, the agent of human malaria [65, 66]. A selective scanning method was used to measure spatially resolved Raman spectra of live *Neospora caninum* tachyzoites colonizing human brain microvascular-endothelial cells. Selective scanning method is based on an algorithm in which the sampling points are not selected in a raster fashion but are determined during the measurements based on the spatial and chemical properties of the sample. This technique allowed the detection of biochemical changes of parasites and their cellular micro-environment with 10-fold shorter acquisition time compared to raster scanning [67]. Atomic force microscopy combined with Raman was used to study bacteria cocultures of mycobacterium and gram-negative bacteria [68]. The combination of quantitative phase microscopy with Raman microscopy imaged the distribution of hemozoin in malaria infected red blood cells [69]. Urinary tract infection (UTI) pathogens were investigated by Raman spectroscopy on a single cell level [70].

2.5 Theory of the Raman Effect

There are two ways to interpret Raman scattering, the classical wave interpretation and the quantum particle interpretation.

In the classical wave interpretation, light is considered as an electromagnetic wave. When an electromagnetic wave interacts with a molecule, it interacts with its electron cloud and induces an electric dipole moment within the molecule;

$$P = \alpha E \quad (1.1)$$

where α is the electric polarizability of the molecule, which is a function of size, shape and chemical bond of the molecule. Larger molecules have higher polarizability values due to large electron clouds. E is the electric field intensity corresponding to the incident light.

$$E = E_o \cos (2\pi\nu_o t) \quad (1.2)$$

where ν_o is the frequency of the laser light and E_o is the electric field amplitude. Thus the electric dipole moment induced in the molecule is;

$$P = \alpha E_o \cos (2\pi\nu_o t) \quad (1.3)$$

The scattering system is considered to be one molecule, which is at equilibrium position q_o , free to vibrate, but not to rotate. The electron cloud of the molecule changes due to the vibration of the nuclei thus the polarizability of molecule changes during the vibration. Thus, the polarizability can be considered a linear function of the normal co-ordinates of vibration. The polarizability can be expanded by Taylor's series for the variation with respect to the normal co-ordinates of vibration.

$$\alpha = \alpha_o + \left(\frac{\partial \alpha}{\partial q} \right) q_o \quad (1.4)$$

where the subscript zero indicates the equilibrium position at which the parameters α_o and q_o are evaluated. Combining these terms and simplifying basic trigonometric identities; the representation of the light scattered by molecule is:

$$P = \alpha_o E_o \cos(2\pi\nu_o t) + \left(\frac{\partial \alpha}{\partial q}\right) q_o E_o [\cos\{2\pi t(\nu_o - \nu)\} + \cos\{2\pi t(\nu_o + \nu)\}]$$

(1.5)

Equation (1.5) [71] demonstrates that the change in polarizability during molecular vibrations leads to light scattering at three frequencies. The first term represents the Rayleigh scattering (elastic scattering) which has a same frequency as that of incident photon ν_o . The second term represents the Raman Scattering (inelastic scattering) of light with frequency shift of $(\nu_o - \nu)$ and $(\nu_o + \nu)$ i.e. gives the Stokes and anti-Stokes scattering of light respectively [72,73,74].

In quantum mechanics the scattering is defined as an excitation of the molecule to a virtual (or forbidden) state lower in energy than a real electronic transition with immediate de-excitation (it occurs in 10^{-14} sec or less) [71,75]. The scattering of light is classified into two categories; elastic scattering and inelastic scattering.

When the scattered light has the same frequency as that of the incident light, this is defined as elastic scattering, also known as Rayleigh scattering. Rayleigh scattering is the most dominant type of scattering. The basic theory of Raman scattering is based on inelastic scattering of light. Figure 2.4 is known as Jablonski diagram. It illustrates the electronic and vibrational energy levels of a molecule and the transitions between ground and excited state when light interacts with a molecule.

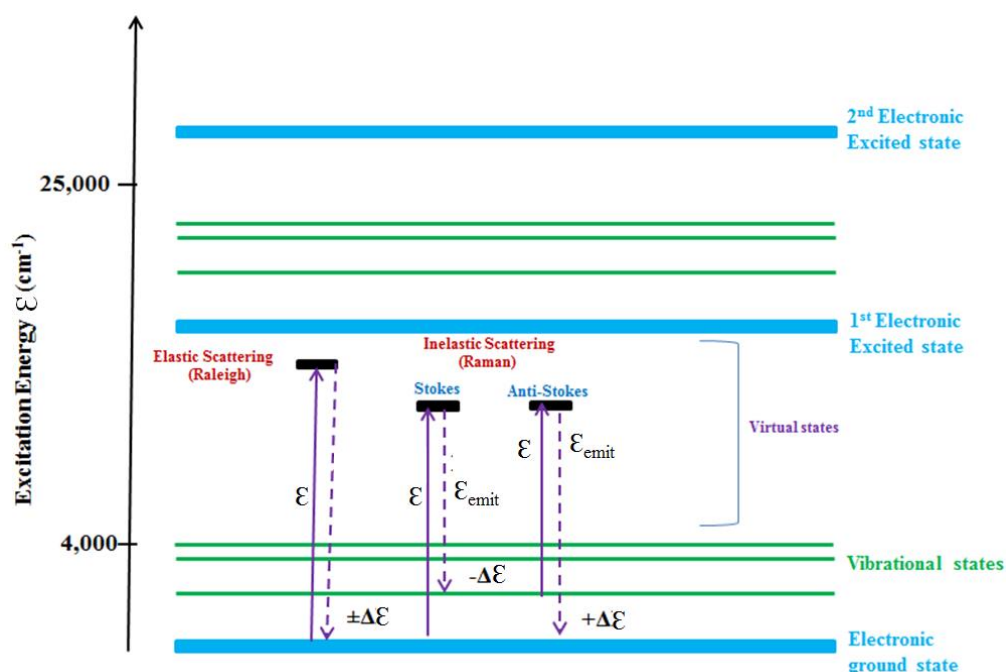


Figure 2.4: Jablonski energy diagram of electronic energy transitions [75].

A small part of the scattered light (approximately 1 in 10 million) [77] is scattered inelastically. The inelastic scattering is known as Raman scattering. In inelastic scattering, the scattered photons have different frequency compared to the incident photons. If a scattered photon has less energy (longer wavelength) than the incident photons, the phenomenon is known as Stokes scattering. If a scattered photon has greater energy (shorter wavelength) than the incident photon, then the phenomenon is known as anti-Stokes scattering. Figure 2.4 illustrates these three phenomena. If initially a molecule is in the ground state and gets excited to virtual state after interaction with the incident photons and drops back on the first vibrational level, then the scattered photon will have the energy of $\mathcal{E} - \Delta\mathcal{E}$ [71] (Stokes scattering). The virtual states are not stationary electronic energy states but virtual intermediate levels to mediate forbidden transitions. If a molecule starts from first vibrational level and get excited to virtual or forbidden state after interaction with the photons and drops back to the

ground vibrational state, then the photon gains $\Delta\mathcal{E}$ energy, this gives energy of $(\mathcal{E} + \Delta\mathcal{E})$ to the scattered photons [71]. This is anti-Stokes scattering.

A Raman spectrum is expressed in units of "Raman shift Δ (cm^{-1})" for the horizontal axis and the "intensity" for the vertical axis. The Raman shift is the frequency difference between the excitation radiation and the Raman scattered radiation and is expressed as:

$$\Delta(\text{cm}^{-1}) = 10^7 \left(\frac{1}{\lambda_o} - \frac{1}{\lambda_R} \right) \quad (1.6)$$

where λ_o is the laser wavelength and λ_R is the Raman radiation wavelength in nanometres. The unit used for wavenumber is ' cm^{-1} ' and it does not depend on the wavelength of excitation laser, therefore it makes easier to compare Raman spectra collected with different excitation sources [71].

In Figure 2.4, the virtual states are the intermediate levels from which the scattering occurs. When the incident radiation leads to actual absorption to an electronic eigenstate, the resultant intense emission is termed fluorescence. The fluorescence can occur only at some specific frequencies, but the Raman effect can occur at any frequency of the excitation photon [71].

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Chapter 3

Methods and Instrumentation

This chapter gives a description of the Raman micro-spectrometer and methods used for spectral calibration relevant to the experimental work on parasites and human cells studies. *T. gondii* is 2-4 μm in diameter, 6-8 μm in length and human cells are 20-30 μm in diameter. However, the internal components of these cells are small and can be approx.1 μm or even smaller. In order to study the biochemical properties of these cells, the Raman microscope is first calibrated for lateral resolution. The Raman micro-spectrometer has also an integrated fluorescence imaging system and details are included in this chapter. The methods for Acridine Orange (AO) staining of nucleic acids (DNA, RNA) in cells are described and the images of healthy and cells infected by parasites are presented.

3.1 Raman micro-spectrometer

The schematic description of the equipment is presented in Figure 3.1. Raman spectra were recorded using a home-built confocal Raman micro-spectrometer optimized for live-cell studies. An inverted microscope (IX71, Olympus, Essex, UK) was selected for the setup as it allowed live cell measurements without additional disturbance of objective dipping in culture media as opposed to up-

right microscopes. The laser was focused through a coverslip at the bottom of a purpose-build sample holder. An environmental enclosure (Solent, Segensworth, UK) was integrated to the microscope to maintain suitable culture conditions (37°C temperature and 5% CO₂) for living cells. Under these conditions, Raman spectral measurements of individual live cells maintained in their original culture media were acquired over several hours without disturbing the cells and by avoiding bacterial contamination. The integration of the environmental chamber with the inverted Raman microscope allowed time- and spatially-resolved molecular studies of cellular processes spanning days and weeks, such as stem cell differentiation [1,2].

Raman scattering is excited by a continuous waveform (CW) Ti:sapphire laser (Spectra Physics 3900S) tuned at 785 nm, with a high beam quality ($M^2 < 1.1$). The laser beam was expanded to over fill the objective aperture, allowing the beam to be focused to a diffraction limited spot. The laser at this near infrared (NIR) wavelength (785nm) was preferred to study live cells because it is less likely to cause cell damage even at high power levels and long exposure times (10-20 minutes) [3]. The use of a laser in the near-infrared region (NIR) also reduces the excitation of fluorescence emission.

The microscope objective used for the live cell measurements was a 60X water immersion objective (Olympus) with a numerical aperture of 1.2. The objective focused the laser on the cell and collected the Raman scattered photons. It had special IR glass for high transmittance in NIR region (>80%). The laser beam was expanded to fill the pupil of the microscope objective (diameter of 7mm) by

placing beam expander in its path, to obtain a laser spot of $0.48\mu\text{m}$. Because Rayleigh scattering is much stronger than Raman scatter light, a dichroic beam splitter (Shamrock, Inc, Rochester, New York, USA) and an ultrasteep longpass Raman filter are used to block the elastic scattered photons [4]. The Raman scattered light passed through the filters and then it was focussed on an optical fibre connected to a spectrometer which separates the incoming light into their corresponding wavenumbers. The Raman scattered light was collimated and then focused on a $50\text{ }\mu\text{m}$ diameter optical fibre connected to a spectrometer (Acton Series LS 785, Princeton Instruments) equipped with a grating of groove density 1200 lines/mm (spectral resolution of $\sim 1.5\text{cm}^{-1}$ in the $600\text{-}1800\text{cm}^{-1}$ region) and cooled deep-depletion back-illuminated CCD detector (Andor Technologies, Belfast, UK). Raman spectral imaging was performed by scanning the cells through the laser focus in a raster pattern (e.g. $1\mu\text{m}$ step size) using a high precision step-motor stage (Prior, Cambridge, UK) and acquiring Raman spectra at each position (usually 1s/pixel).

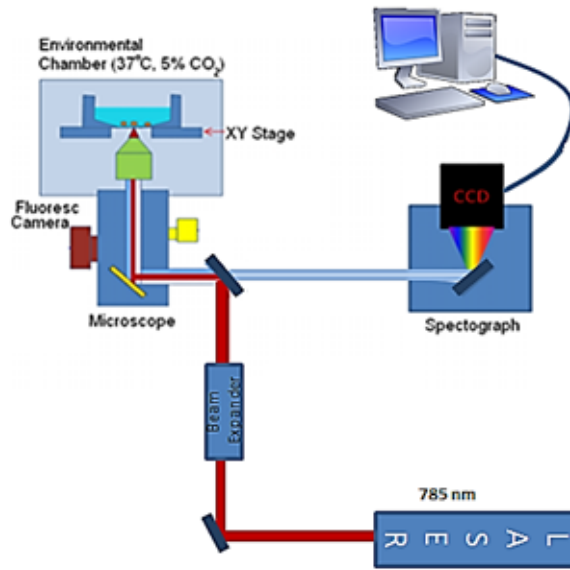


Figure 3.1: Schematic description of the Raman micro-spectrometer [5]. Sample holder is placed on microscope stage enclosed in an environmental chamber (37C°, 5% CO₂). The setup of a Raman microspectrometer consists of a 785nm laser as an intense and monochromatic light source, beam expander, objective, filters that separates the elastically (Rayleigh) scattered light of the sample from the Raman (inelastically) scattered light, a spectrograph, and a detector. Information is passed from detector to the computer.

3.2 Spectral calibration

The Raman spectrometer was calibrated for the wavenumber axis before the start of the measurements on cells. In this respect, American Society for Testing and Materials (ASTM) has chosen eight materials as Raman shift standards (ASTM E 1840) [6]. Here, 4-acetamidophenol (the active ingredient of Tylenol) (Sigma-Aldrich) and polystyrene (Sigma-Aldrich) were used for calibration. The spectral peaks of these chemicals are indicated by the ASTM standards as shown in Figure 3.2. For wavenumber axis calibration of the spectrometer, a third or fourth-order polynomial was used to fit the observed detector output to the known standard Raman shifts with the calculated mean square error (MSE) of

less than 0.5cm^{-1} . In the next step, another sample from one of these materials was selected and measured with the system and plotted with the calibrated wavenumber axis. An example of the calibrated Tylenol spectrum with an accuracy of peak position of 0.5cm^{-1} is presented in Figure 3.3.

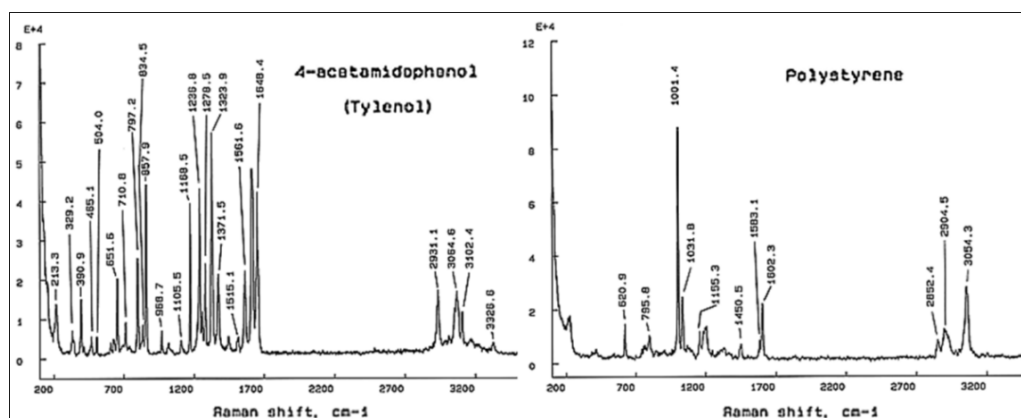


Figure 3.2. Demonstration of Raman spectra with peak assignments of calibration chemicals: 4-acetamidophenol (Sigma-Aldrich) and polystyrene (Sigma-Aldrich) according to ASTM E 1840 [6].

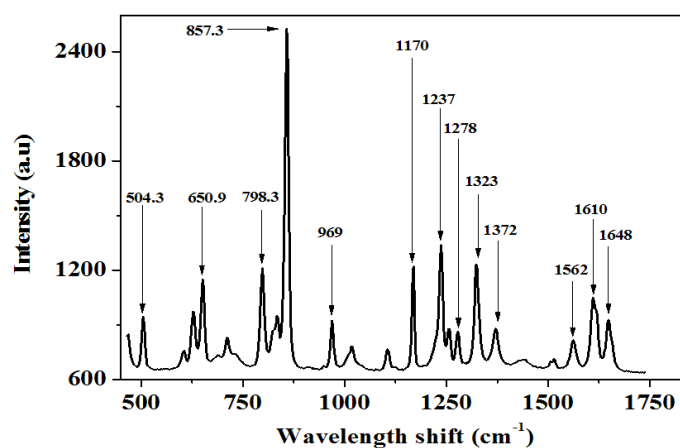


Figure 3.3 Raman spectrum with peak assignments of a 4 acetamidophenol (Tylenol) (Sigma-Aldrich), after spectral calibration.

3.3 Spatial Resolution

The spatial resolution determines the ability of a microscope to resolve the spatial features of the imaged object. Here, spatial resolution was measured only in the lateral (x-y plane) dimensions.

Lateral resolution defines the separation between two point-like objects that can be distinguished by the instrument, as shown in Figure 3.4a. If the points cannot be distinguished, they appear as a single larger spot. The lateral resolution is determined by calculating the FWHM of the peak plotted for the peak intensity change from one end to the other end of the point object. The separation between peaks corresponding to the point objects determines the resolution between them as shown in figure 3.4b.

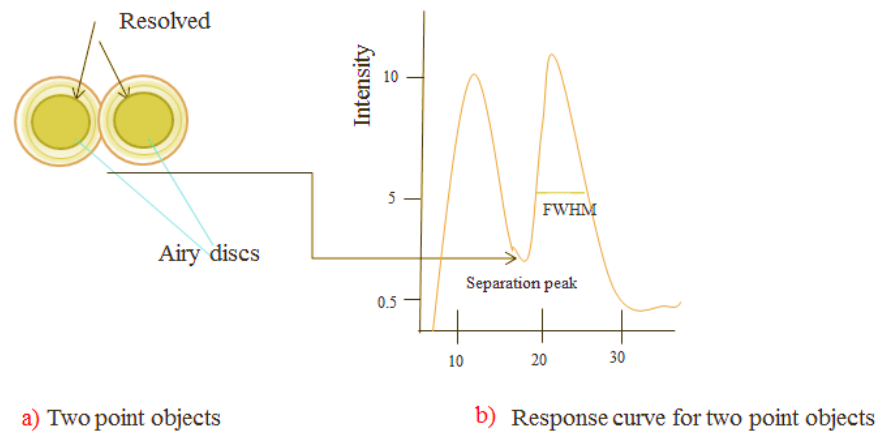


Figure 3.4: Theoretical representation of two point objects to explain the Airy disc and intensity profile.

The theoretical model of the resolution is based on the mathematical model of Airy discs caused by diffraction of light. The small point object is considered to be the central bright ring with progressively concentric dark and bright rings.

The r_{airy} is the radius of first dark ring that depends on wavelength (λ) of the illumination source of object and numerical aperture (N.A) of the objective and is defined as;

$$r_{airy} = 0.61 \frac{\lambda}{N.A} \quad (3.1)$$

$$N.A = n \times \sin \mu \quad (3.2)$$

where N.A is the numerical aperture of the microscope objective and μ is the maximal half-angle of the cone of light that can enter or exit the objective.

The Rayleigh criterion states that two points can be resolved if the distance 'd' between them is greater or equal to the Airy radius. The calculated Airy radius for the Raman microscope was 399nm (N.A 1.2 and $\lambda=785\text{nm}$).

The lateral resolution of the instrument was measured experimentally by collecting Raman spectral maps for 1 μm diameter polystyrene beads (Sigma Aldrich). The maps were collected by raster scanning of the polystyrene beads with step size of 0.1 μm to 1 μm . Due to a CCD artefact at the band 1001cm^{-1} , the area of the spectral band 1602.3cm^{-1} was selected for producing Raman maps as shown in Figure 3.5. The individual beads started to become distinguishable at 0.5 μm step size. The lateral resolution was calculated as the FWHM of the profile curves obtained from the lines A, B, C as the area of the selected peak changes from one end to the other end. The lateral resolution was found to be approximately 2 μm for 0.5 μm step size as shown in Figure 3.6.

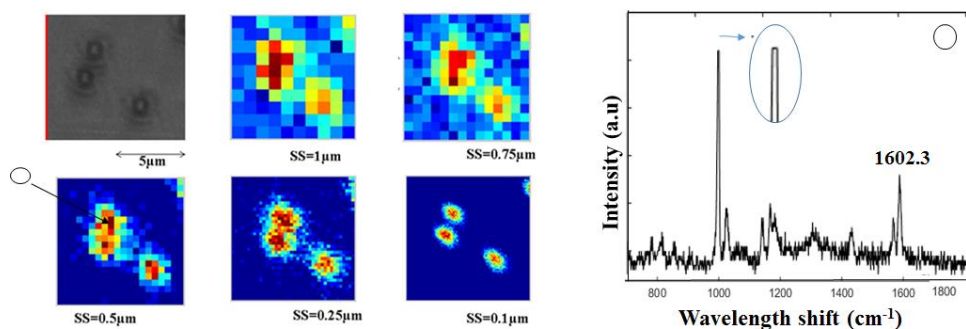


Figure 3.5: Optical image and Raman spectral maps of the polystyrene beads (1 μm diameter) measured with different step sizes (SS). Polystyrene spectrum is shown at right from polystyrene bead 0.5ss and 1sec time of integration A typical spectrum of a polystyrene bead is shown on right (1sec integration time).

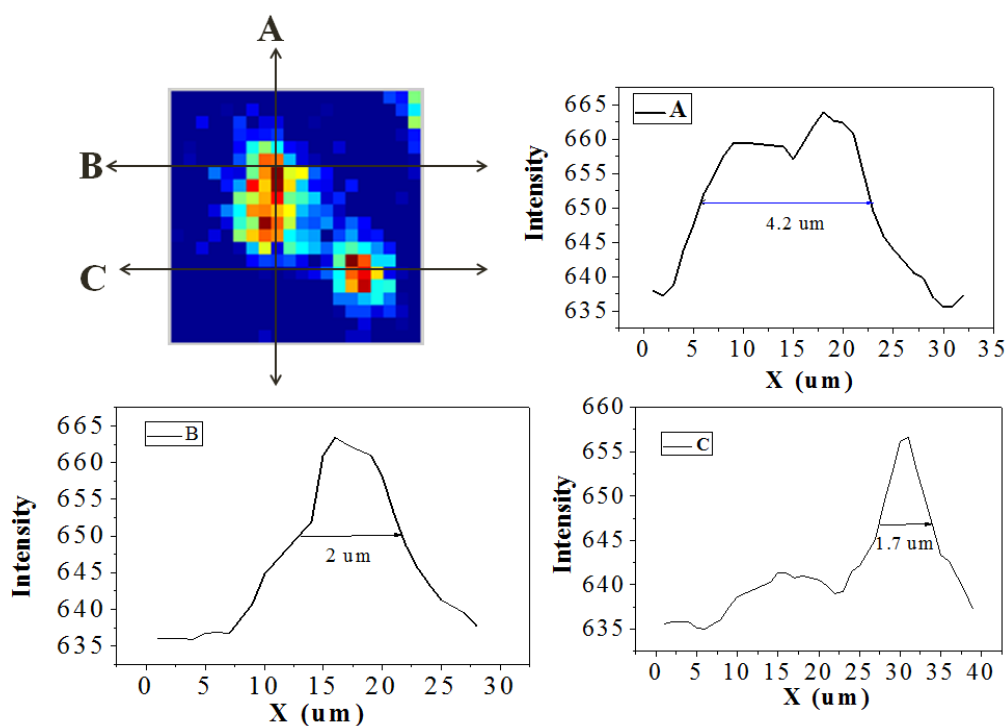


Figure 3.6: Raman spectral image of 1 μm diameter polystyrene beads scanned with 0.5 μm step size. Profile curves of spectrometer produced in the X and Y plane on the lines A, B, C (the peak area at 1602.3 cm⁻¹). The lateral resolution is calculated by the FWHM of the profile curves obtained from A, B, and C lines.

3.4 Fluorescence Imaging

Fluorescence staining is a well-established technique in cell biology and involves the staining of cellular molecules with specific dyes. For example, DAPI (4', 6-diamidino-2-phenylindole) can be used to label the DNA in a cell. Here we used the fluorescence imaging to initially validate the results obtained by Raman micro-spectroscopy. A wide field fluorescence imaging system was integrated on the Raman microscope, which included an Hg lamp, a sensitive CCD camera and a set of fluorescence filter cubes (Table 3.1). A fluorescence filter cube contains a set of filters combination housed in an optical block. It consists of one excitation filter, an emission filter and a dichromatic beam splitter/mirror as shown in Figure 3.7. Excitation filter allows only specific wavelength of light to pass through to excite the molecules in the sample. A dichroic beam splitter is introduced after the excitation filter and before emission filter at an angle of 45° to both. It acts as a mirror for excitation wavelength of light and reflects it to the sample. The emission filter separates the fluorescently emitted light from fluorescent background and dichroic mirror transmits it to the fluorescent detector. The combination of wavelengths of the filters and mirror in a cube are selected according to the fluorophore's excitation and emission wavelengths range used in wide-field fluorescent microscopy. The filter cubes used in our system are given the Table 3.1.

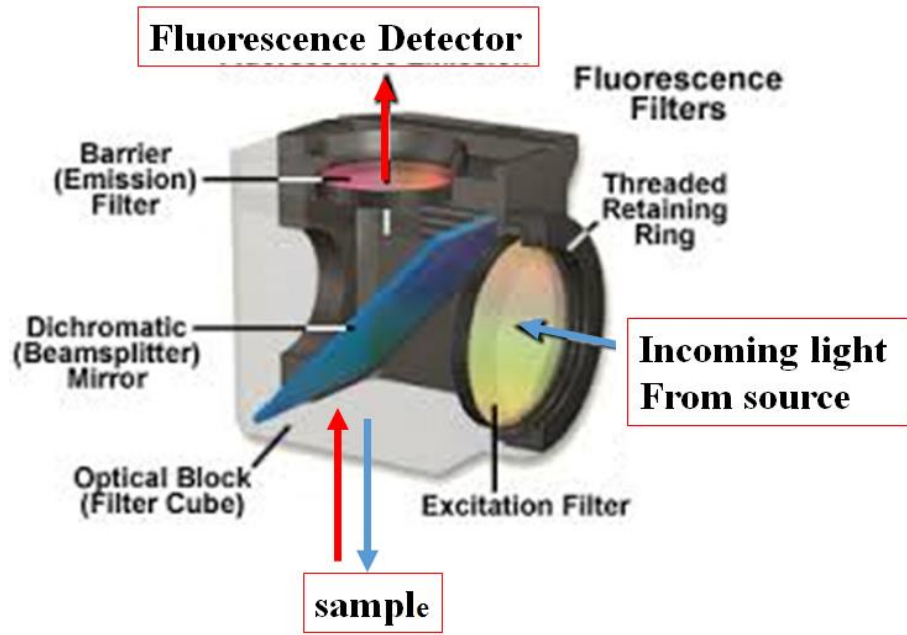


Figure 3.7: Filter cube with pre-mounted Excitation filter, emission filter and dichroic mirror [7].

Filter Cube	Excitation Wavelength Range/ Bandwidth (nm)	Emission Wavelength Range/ Bandwidth (nm)	Dichroic (nm)
TRITC	542 / 20	620 / 52	570 (edge)
FITC	475 / 35	530 / 40	499 (edge)
DAPI	360 / 10	420	412
CY3	530/40	593/40	562

Table 3.1: Details of pre-mounted filter cubes in the Raman microscope.

3.4.1 Calibration of the fluorescence imaging system

Prepared fluorescent microscope slides are available to calibrate the fluorescence microscope setup. A FluoCells® Prepared Slide #1 was purchased from Life Invitrogen [8]. The calibration slide had a 16 μm cryostat section of mouse kidney sample, consisting of BPAE (Bovine Pulmonary Artery Endothelial) cells, multilabeled with MitoTracker® Red CMXRos, Alexa Fluor® 488 Phalloidin and DAPI. The MitoTracker Red, a red-fluorescent stain was used for imaging the mitochondria inside cells and Alexa Fluor® 488 phalloidin for F-actin in the cytoplasm, while the nuclei were stained with the blue-fluorescent DNA stain DAPI [8].

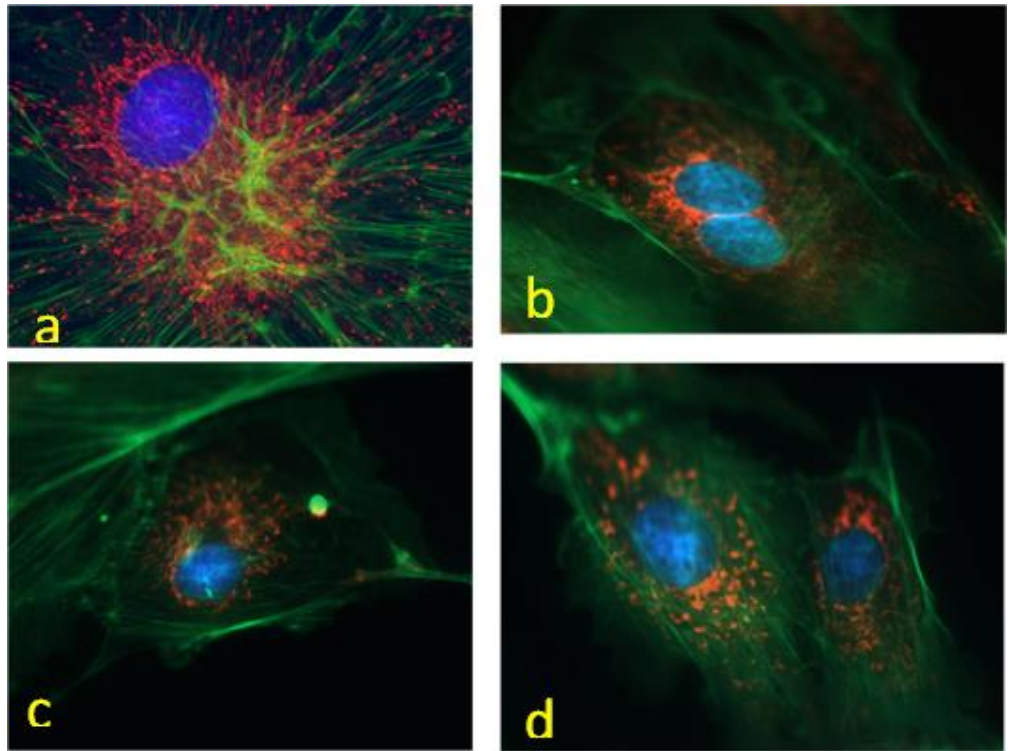


Figure 3.8: (a) Standard image of the Invitrogen calibration slide [8], (b, c, and d) images of the calibration slide acquired with the fluorescence imaging system on the Raman microscope.

The image of the calibration slide presented in Figure 3.8(a) was provided by Invitrogen, where images 3.8(b, c, d) were obtained with the fluorescence imaging system on the Raman microscope. By comparing the images "b, c, d" with image "a" in Figure 3.8, it is concluded that Raman microscope provide very high quality fluorescence images of cellular components.

3.4.2 Fluorescence staining and imaging of cells

Fluorescence images were defined for cells on glass, MgF₂ and quartz coverslips. As glass slides cannot be used for Raman cell imaging due to the high background, MgF₂ and quartz cover slips were used in our cell chambers for Raman and fluorescence imaging.

Cells were grown on glass slides, and in the titanium sample holders containing MgF₂ and quartz coverslips. Half of the prepared healthy cells were infected with parasites (*T. gondii*), and at 48 hours post infection, cells were fixed and stained with AO to image parasites inside the cells.

Acridine orange (AO) staining was used to distinguish the morphological appearance of the intracellular developing *T. gondii* tachyzoites from host cell organelles. AO is a DNA intercalating dye and is used to differentially stain RNA (Ribonucleic acid) and DNA (Deoxyribonucleic acid) inside eukaryotic cells. AO staining gives green colour to DNA in nucleus and red colour to RNA inside cytoplasm. Cells were fixed in 4% paraformaldehyde for 15 min, followed by washing with 1x PBS twice. Fixed cells were incubated with 5% AO staining solution for 10 min and fluorescent imaging of the cells has been carried out afterwards. The images are shown in Figure 3.9.

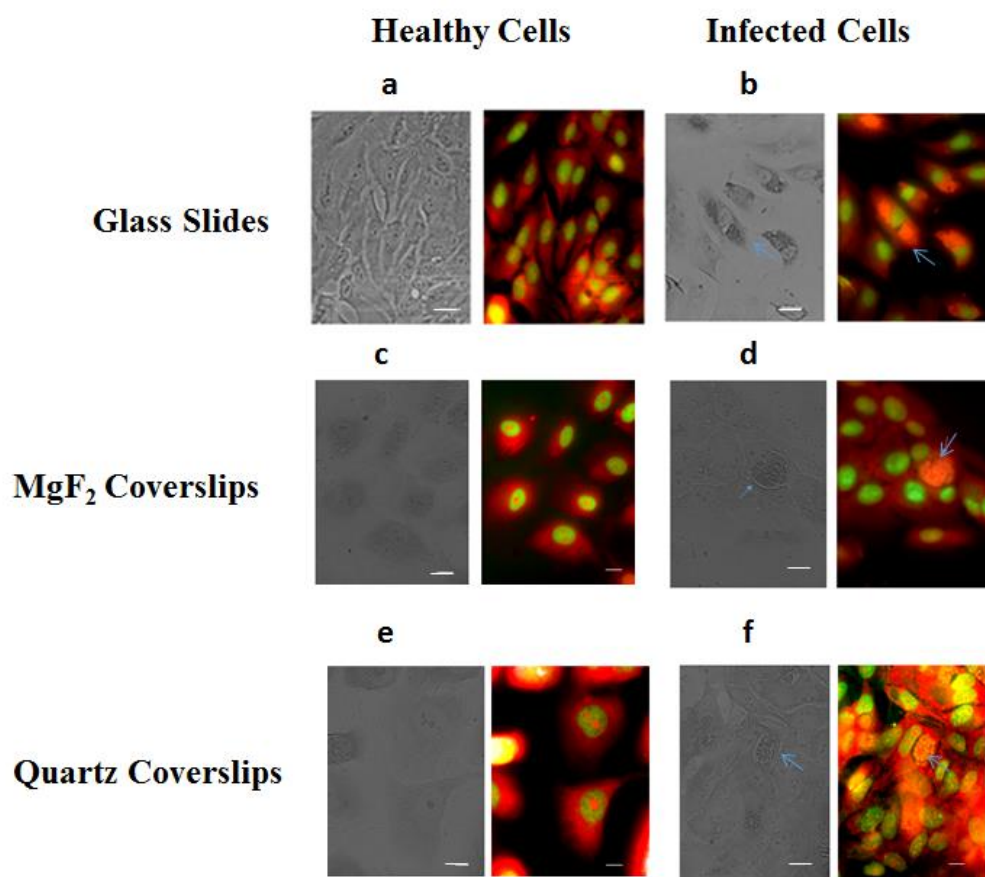


Figure 3.9: Fluorescence images (AO staining) of healthy human cells and cells infected with *T. gondii*. Cells were cultured on glass, MgF₂ and quartz coverslips.

MgF₂ coverslips were used for Raman and fluorescence imaging in the work presented in the experiments given in the Chapter 4 and 6. Quartz coverslips were selected for Raman and fluorescence imaging in the work presented in the Chapter 5, because they give very bright and sharp fluorescence images due to higher refractive index 1.4585 compared to MgF₂ 1.38-1.37 in the region of interest.

3.5 Spectral data pre-processing and analysis

Data pre-processing and analysis was done by using in-house built functions in Matlab (The MathWorks, Natick, MA). Data pre-processing involves cosmic

ray removal, background subtraction and noise reduction of the data. Cosmic rays are the very sharp and narrow lines appear in the measured spectra. They are generated by hitting of the random high energy particles to the detector. The cosmic ray removal algorithm is designed to detect where the gradient is 10 standard deviations above from the average gradient of the Raman spectrum. This semi-automatic program is applied on each spectrum of the measured data set to remove the cosmic ray peak and substitute it with a mean intensity of the two edges of the deleted peak. To increase the signal to noise ratio, Singular value decomposition (SVD) has been used [8].

The background signal is because of the non-Raman photons recorded by the detector. It originates due to the optics of the microscope, and the stray laser light. The background measurement is based on 10 measurements of $10\ \mu\text{m} \times 10\ \mu\text{m}$ areas between the cells, and then calculating the mean background spectrum. Background is subtracted from each spectrum of the data.

For the data Analysis, univariate and multivariate methods have been used. Univariate method used are based on the peak intensity and ratio of the intensities of pre-chosen bands. The multivariate unsupervised PCA (Principal component analysis) has been used to see the differences and similarities in the large data sets. PCA transforms a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components [9,10]. The results of the PCA are represented in term of scores and loadings.

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Chapter 4

Analysis of interaction between the apicomplexan protozoan *T. gondii* and host cells using label-free Raman Spectroscopy

In this chapter, label-free imaging using Raman micro-spectroscopy (RMS) was used to characterize the spatio-temporal molecular changes of *T. gondii* tachyzoites and their host cell microenvironment.

4.1 Introduction

Human retinal cells were infected with *T. gondii* tachyzoites and Raman spectra were measured from the parasites and the surrounding cytoplasm of the host cells. Initial experiments were performed on paraformaldehyde-fixed cells, to provide an overview of the molecular changes, followed by measurements on live cells to demonstrate the potential of Raman microscopy for label-free time-course analysis. Raman spectral maps were recorded from isolated *T. gondii* tachyzoites and *T. gondii*-infected human retinal cells at 6 hr, 24 hr and 48 hr post-infection. Principal component analysis (PCA) of the Raman spectra of paraformaldehyde-fixed infected cells indicated a significant increase in the amount of lipids and proteins in the *T. gondii* tachyzoites as the infection progresses within the host cells. These results were confirmed by experiments

carried out on live *T. gondii*-infected cells and were correlated with an increase in the concentration of proteins and lipids required for the replication of this intracellular pathogen. Our findings reveal previously unrecognized chemical features of *T. gondii* tachyzoites interacting with host cells and suggest that these biomolecules contribute to *T. gondii* invasion and parasite virulence during infection.

4.2 Materials and Methods

4.2.1 Cell culture

The human retinal pigment epithelial cell line (ARPE-19) was kindly provided by Dr Kevin Webb and Emilia Moradi, University Of Nottingham. Cells were maintained *in vitro* in Dulbecco's modified Eagle's medium, low glucose, pyruvate (DMEM, Life technologies, Grand Island, NY) supplemented with 1% penicillin/streptomycin (Invitrogen Corp., Carlsbad, CA) and 10% heat-inactivated fetal bovine serum (FBS, Invitrogen Corp., Carlsbad, CA). As an immune-privileged and easily accessible organ, the eye constitutes a favourable target for *T. gondii*. Hence, we selected this cell line as a model to examine the injury caused by *T. gondii* infection to the eye.

4.2.2 Growth and preparation of parasites

T. gondii tachyzoites were maintained *in vitro* by serial passage on monolayers of human brain microvascular endothelial cells (HBMECs) at 37°C in 5% CO₂. HBMECs were grown as described previously [1]. Infected host cell monolayers were scraped, parasites were isolated from host cells by passage through 25- and

27-gauge needles and purified by using PD-10 Desalting Columns prepacked with Sephadex G-25 medium as described previously [2]. Purified parasites were centrifuged at $800 \times g$, washed twice with fresh cRPMI, re-suspended in fresh medium and quantified using a hemacytometer. The final volume of suspension was adjusted with cRPMI medium to achieve a ratio of 2:1 parasite/host cell for subsequent infection experiments. Parasite viability was checked by using trypan blue staining assay and parasites with more than 97% viability were used.

4.2.3 *In vitro* infection protocol

ARPE-19 cells (3×10^5 cells/mL) were seeded in titanium cell-chambers that fitted 6-well culture plates with a volume of 2 mL cRPMI medium/well. The titanium cell chambers were purpose-built to enable acquisition of Raman spectra of the cells and incorporated MgF₂ coverslips (0.17 mm thick) at the bottom. The cells were allowed to grow overnight by incubation at 37°C in a humidified atmosphere with 5% CO₂ in air. Before infection, cell growth medium was removed and cells were washed three times with sterile PBS (8 g/L NaCl, 0.2 g/L KCl, 0.2 g/L KH₂PO₄, 1.15 g/L Na₂HPO₄). Then, in each chamber parasites were added at a MOI of 2 in 2-ml fresh medium, and the control chambers received only 2-ml fresh medium (mock-infected). Culture plates were then incubated to allow infection to progress within cells. Parasite and control cultures were sampled at different time points post infection (PI) starting from 0 h, and then, at 6, 24 and 48 h PI. At each sampling time two chambers (one infected and one control) were collected from the 6-well culture plate and subjected to Raman Spectroscopy imaging.

4.2.4 Raman spectroscopy

A 785 nm ~200 mW laser (before objective) (Ti:sapphire laser, Spectra-Physics) was used for excitation of Raman spectra. Raman spectral imaging was performed by scanning areas of the cells through the laser focus in a raster pattern (1 μm step size, typically areas of $15\mu\text{m} \times 15\mu\text{m}$ at 6 hours PI up to $40\mu\text{m} \times 60\mu\text{m}$ at 48 hours PI) and acquiring Raman spectra at each position (2 s/pixel). The schematic diagram and description of Raman micro-spectrometer system is shown in Figure 3.1 in chapter 3. The retrospective positioning and identification of the cells was based on two thin marks engraved on the cell chambers (retro-positioning accuracy was 5 μm). The spectrometer wavenumber axis was calibrated prior to each experiment using Tylenol sample indicating an accuracy of 0.5 cm^{-1} .

4.2.5 Fluorescence imaging

AO staining and fluorescence imaging of Healthy and *T. gondii*-infected cells were performed, on the same cells, after completion of the Raman spectral imaging. AO staining gives green colour to DNA in nucleus and red colour to RNA inside cytoplasm. The method of the staining is explained in Chapter 3, section 3.4.2.

4.2.6 Data analysis

The spectra are collected from cytoplasm of control and Infected cells. Data pre-processing involved cosmic ray removal, background subtraction. After the pre-processing of data, Raman image and fluorescent images of the measured cells

were overlapped by using image registration in matlab programme. After this, the spectra were picked up from the cytoplasm/parasite area by creating freehand region in matlab. By using these methods, spectra from cytoplasm of control cells, spectra from cytoplasm of infected cells, spectra from parasite area of infected cells, isolated parasites were collected. The resulting Raman spectra were analysed by principal component analysis (PCA). The PCA model was established then using the Raman spectra of fixed cells. This model was then applied to calculate the scores for the Raman spectra corresponding to live cells. The scores for PC2 were analysed by Student t-test.

For the Raman spectra presented in Figures 1, 3, 4, 5, and 7, a noise reduction algorithm was applied based on PCA. All Raman spectra were reconstructed based on the first 10 PCs [3,4]. All spectra were normalised to zero mean and unit variance before plotting in the figures.

4.3 Results and Discussion

4.3.1 Raman spectra of human retinal cells and *T. gondii*

Figure 4.1 shows typical examples of Raman spectra and molecular images for healthy human retinal cells and isolated *T. gondii*. For the retinal cells, fluorescence images corresponding to acridine orange (AO) staining were acquired after the completion of the Raman spectroscopy measurements. The stain was taken up by both parasite-infected and control cells.

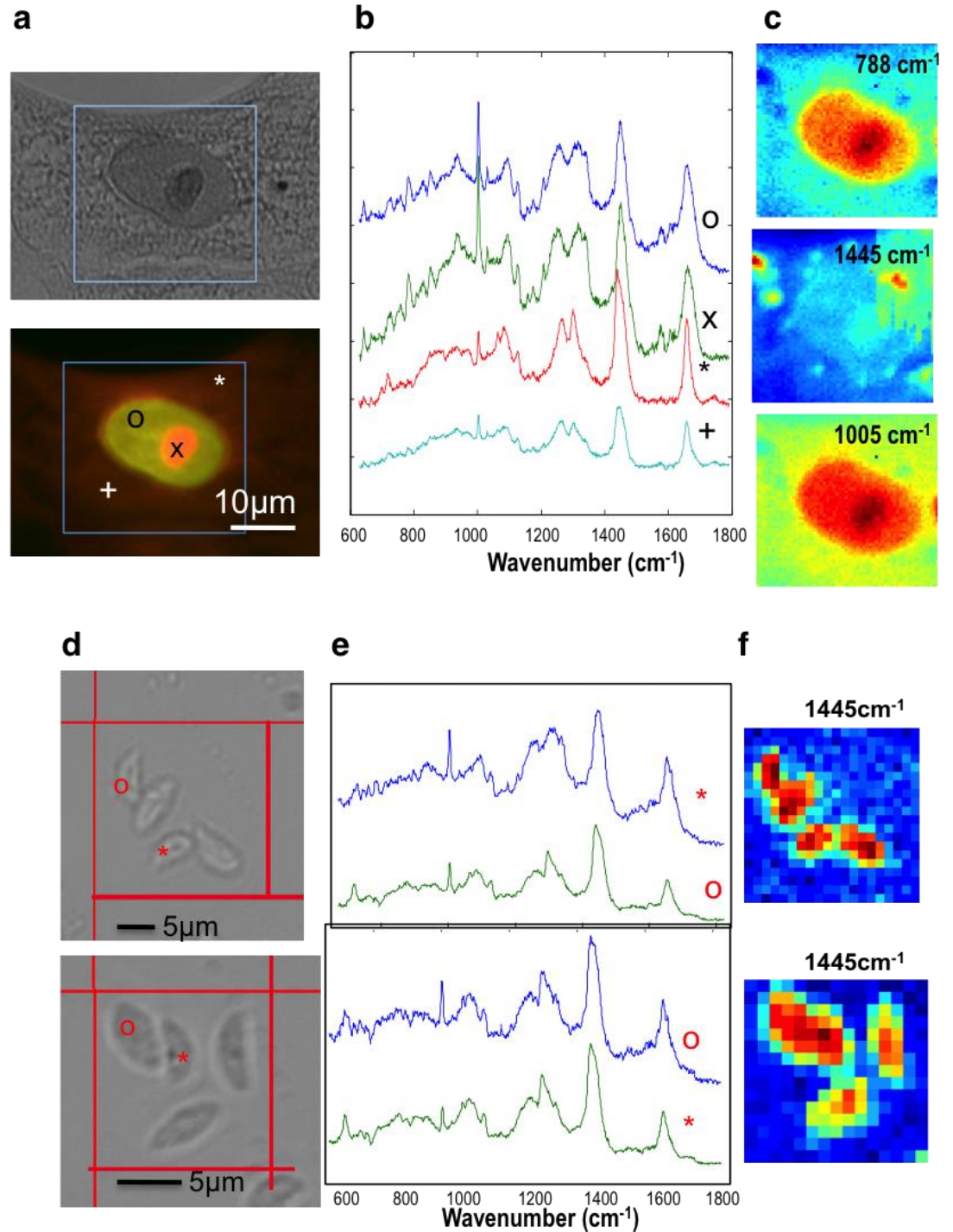


Figure 4.1: Raman spectra and label-free molecular imaging of healthy human retinal cells and isolated *T. gondii* tachyzoites. (a) Brightfield (top panel) and acridine orange fluorescence (bottom panel) images of a typical retinal cell. Fluorescence images are obtained after completion of the Raman spectroscopy measurements. (b) Typical examples of Raman spectra acquired at different locations inside the cell. (c) Raman images obtained using the peak intensity of the 788 cm^{-1} (nucleic acids), 1445 cm^{-1} (lipids & proteins), and 1005 cm^{-1} (proteins) bands. (d) Bright field image of isolated *T. gondii*. (e) Selected Raman

spectra of *T. gondii* tachyzoites. (f) Raman images obtained using the peak intensity of the 1445 cm^{-1} band (lipids & proteins).

Fig. 4.1b shows that the Raman spectra measured from the cell nuclei elicit intense bands corresponding to nucleic acids (O-P-O 788 cm^{-1} , PO_2^- 1098 cm^{-1} , ring vibrations of G and A 1578 cm^{-1}) and proteins (phenylalanine 1005 cm^{-1} , tyrosine 830 cm^{-1} and 850 cm^{-1} , amide I 1660 cm^{-1} , amide III $1200\text{-}1400\text{ cm}^{-1}$, CH_2 deformation 1449 cm^{-1}), with the highest intensity measured in the nucleolus.

Bands corresponding to proteins are also present in the Raman spectra of cytoplasm, but the most intense bands are assigned to lipids: C=C stretching 1660 cm^{-1} , CH_2 deformation 1445 cm^{-1} , CH_2 twisting 1303 cm^{-1} , =C-H deformations 1267 cm^{-1} , O-P-O symmetrical stretch and acyl back bone C-C bands at 1090 cm^{-1} and 1062 cm^{-1} , symmetric stretching of $\text{N}(\text{CH}_3)_3$ of choline group 719 cm^{-1} . Micrometric regions rich in lipids can be identified in the cytoplasm, corresponding probably to lipid bodies. The Raman spectra of isolated fixed *T. gondii* (Figures 4.1d, 4.1e) are dominated by intense bands corresponding to molecular vibrations of lipids and proteins. The spectra of *T. gondii* have higher intensity-ratio between the CH_2 twisting 1303 cm^{-1} and =C-H deformations 1267 cm^{-1} bands compared to healthy retinal cells, suggesting a higher concentration of saturated lipids in *T. gondii*. It is also interesting to note that only small contribution from nucleic acids was detected in the Raman spectra of isolated *T. gondii*.

4.3.2 Time-course study of pathogen-host interaction using fixed cells in parallel cultures

One of the key challenges when measuring Raman spectral maps of live cells is the potential changes in cell morphology during the typically long acquisition times of raster-scanning (20-40 min). Such morphological changes make it difficult to compare the Raman maps with images obtained by other label-based modalities in order to identify the exact locations in a cell where the spectra were measured. Thus, prior to performing time-course experiments on live cells, Raman spectroscopy mapping by raster-scanning was carried out on cells fixed with 4% ice-cold paraformaldehyde. After Raman mapping, the cells were stained with acridine orange, allowing identification of the *T. gondii* growing in the retinal host cells (red emission for RNA mainly in the cytoplasm and green emission for DNA in the nuclei). Measurements were carried out on cytoplasmic regions of control (non-infected) human retinal cells and from *T. gondii* tachyzoites within the host cells at 6 hr, 24 hr and 48 hr post infection (PI). Ten cells were measured at each type point. To provide a statistical analysis of the Raman spectra that would account for both intra- and inter-cellular variability, all spectra were included into a single dataset and were analysed by principal component analysis (PCA).

Fig. 4.2a presents the loadings of the first six principal components accounting to 58% of the total variance of the spectra (component seven was dominated by noise). While the loadings corresponding to PC1 and PC3 consisted only of broad bands characteristic to the background water, the loadings corresponding to PC2, PC4, PC5 and PC6 consisted of bands that can be correlated to specific molecular vibration in biomolecules. PC2 (14.88% variance) is dominated by

strong bands corresponding to proteins and lipids. PC4 (2.20% variance) and PC5 (1.18% variance) have bands mostly associated to lipids, while PC6 (0.79% of variance) has positive bands at 788 cm^{-1} and 813 cm^{-1} that can be associated to nucleic acids.

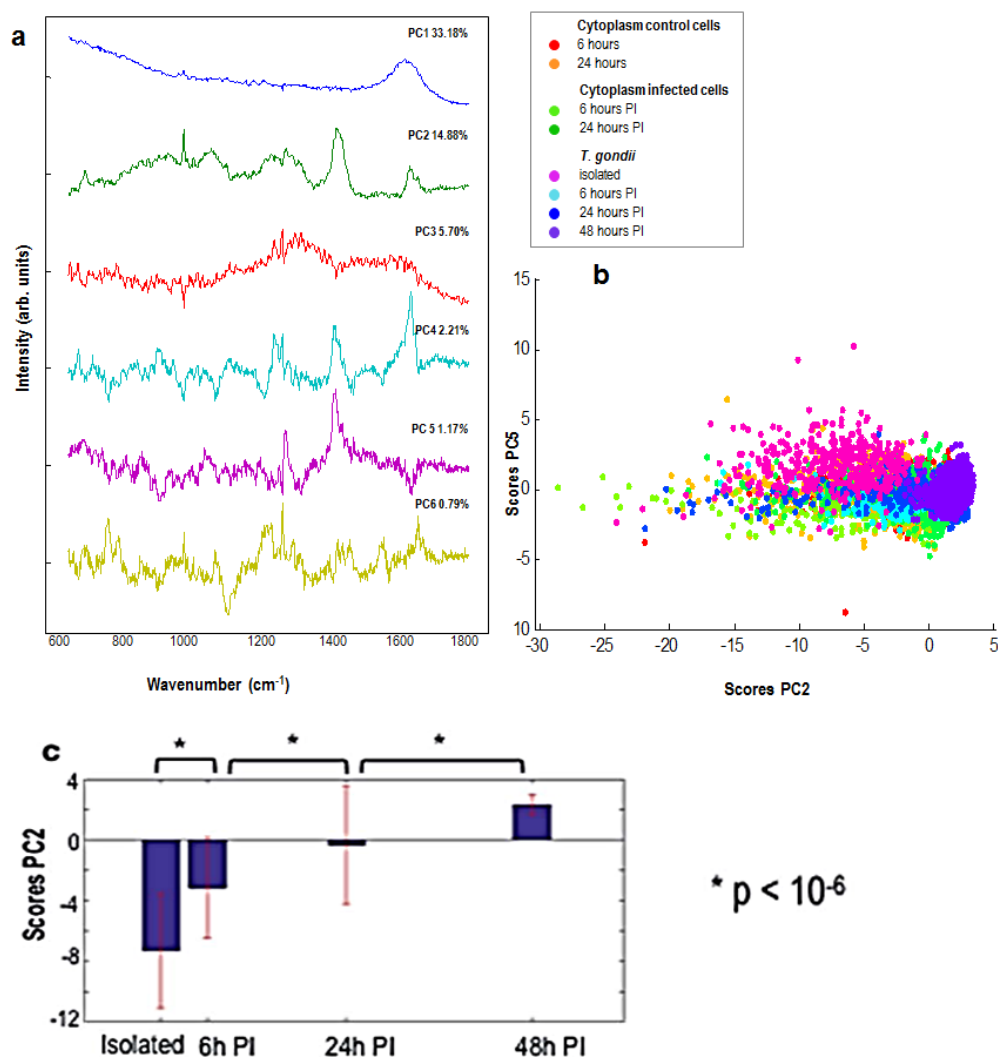


Figure 4.2: Principal component analysis (PCA) of the Raman spectra collected from control human retinal pigment epithelial cell line (ARPE-19) cells, isolated *T. gondii* parasites, *T. gondii* inside ARPE-19 host cells (6 h, 24 h, 48 h PI), cytoplasm of the ARPE-19 cells in the vicinity of the *T. gondii* (6h, 24 h PI). (a) PCA loadings. (b) Scatter plot PC2 vs. PC5 for all spectra; (c) PC2 scores of isolated *T. gondii* and *T. gondii* in host cells, * represents $p < 10^{-6}$ for t-test, error bars represent standard deviations.

The increase in PC2 scores with the infection time indicates an increase in protein and lipid concentration in *T. gondii* during infection. Inspection of the scores related to the other principle components indicates that these components capture only inter- and intra-cellular variability related to lipids and nucleic acids, but no trends related to *T. gondii* infection can be observed. Figure 4.2b presents 2-D plots corresponding to the principal component scores. The results show that PC2 captures most spectral changes related to the *T. gondii* developing inside the host ARPE-19 retinal cells. Although the PC2 scores for the Raman spectra of isolated *T. gondii* tachyzoites and cytoplasm regions of the healthy retinal cells (control) vary within a broad range reflecting the inter- and intra-cellular variability, Figure 4.2c shows that the PC2 scores corresponding to the spectra of *T. gondii* increase as soon as 6 h PI to form a distinct cluster at 48 h ($p < 10^{-6}$ in t-test).

To obtain a better understanding of the molecular changes captured the PCA, Raman maps corresponding to the principal components scores were created. Figure 4.3a shows that at 6 h after the addition of *T. gondii* tachyzoites to the retinal cell cultures, typically only 1–2 parasite cells were found inside the host cells. The PC2 maps indicate higher scores at the locations of the *T. gondii* and the Raman spectra measured at these locations, indeed, show intense bands assigned to lipids and proteins. The Raman spectra of *T. gondii* inside the host cells also show bands corresponding to nucleic acids (788 cm^{-1}), bands which were not detected in the isolated *T. gondii* tachyzoites. These changes may correlate to the increase expression of RNA required for protein synthesis during the growth of the *T. gondii*. After 24 h PI, the *T. gondii* tachyzoites multiplied

inside the host cells and typically 3–4 parasites can be observed in the bright field and fluorescence images (Figure 4.4). The scores corresponding to the PC2 increases compared to 6 h and the Raman spectra indicate intense bands assigned to lipids and proteins. The maps corresponding to the PC6 also show an increase of nucleic acids.

At 48 hr PI, *T. gondii* formed a large vacuole consisting of more than 10 tachyzoites that occupied almost the entire cytoplasm of the host cells. The nuclei of the host cells are squeezed at the edges of the cells. The PC2 scores reached the highest values indicating the increase in protein and lipid synthesis during infection. The maps corresponding to the PC6 scores correlate to the locations of the nuclei of both host and *T. gondii* tachyzoites, however the intensity of the 788 cm^{-1} and 1098 cm^{-1} bands are similar to the intensities at 6 hr and 24 hr PI. In addition, the PC4 maps at 48 hr PI show an increase in the PC4 scores in the host cytoplasm surrounding the parasite vacuole, suggesting an increased concentration of unsaturated lipids. Selected Raman spectra at these locations indicate an increase in the intensity ratio of the 1303 cm^{-1} and 1273 cm^{-1} bands.

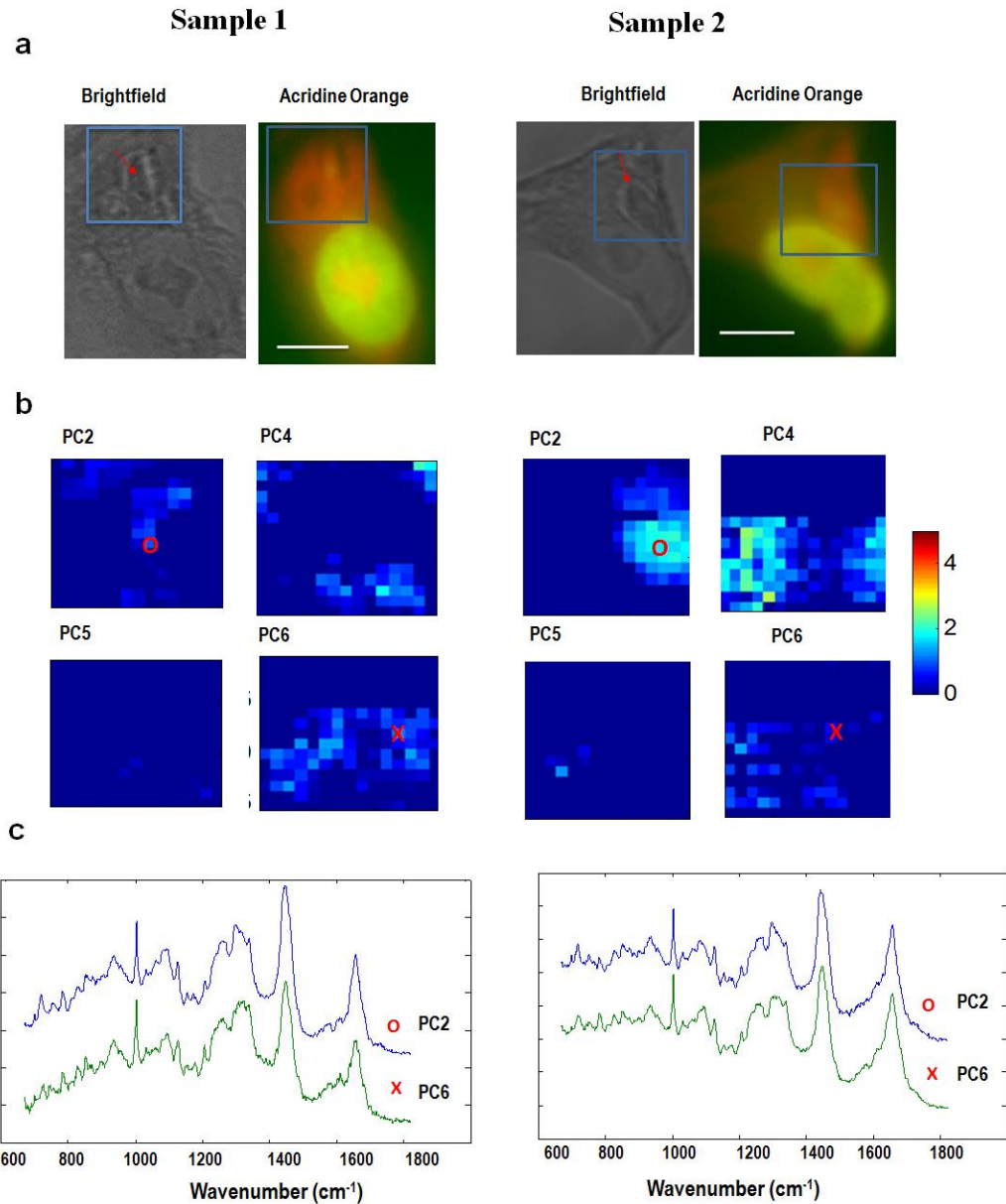


Figure 4.3: Raman analysis of *T. gondii* infected cells at 6 hr PI. (a) Bright field and fluorescence images (recorded after Raman spectroscopy measurements). Red arrows show the position of parasite inside host cell cytoplasm (b) Maps corresponding to principal components PC2, PC4, PC5 and PC6. (c) Selected Raman spectra at locations indicated by o, x symbols. Scale bars: 10 microns.

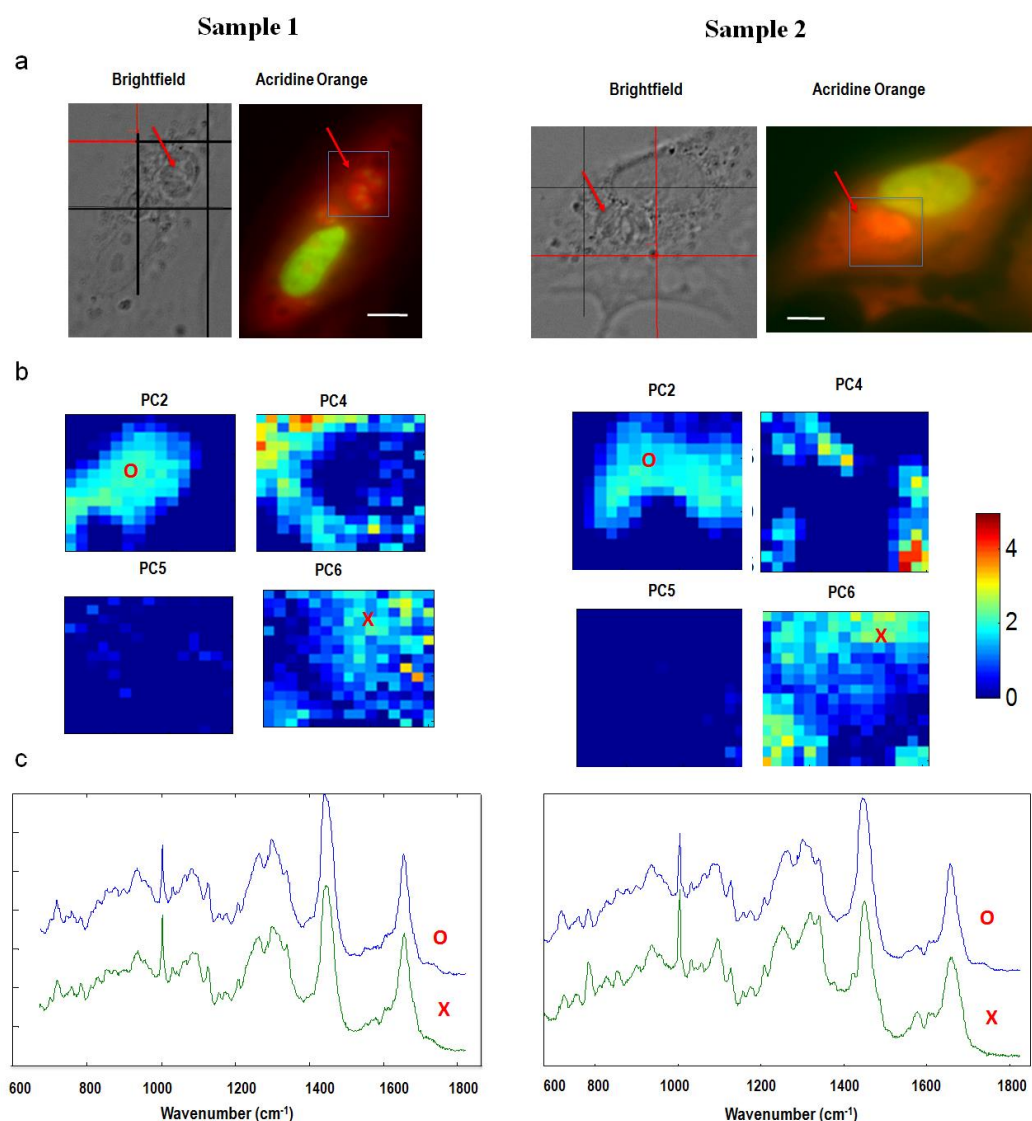


Figure 4.4: Raman analysis of *T. gondii* infected cells at 24 hr PI. (a) Bright field and fluorescence images (recorded after Raman spectroscopy measurements), Red arrows show the position of parasite inside host cell cytoplasm. (b) Maps corresponding to principal components PC2, PC4, PC5 and PC6. (c) Selected Raman spectra at locations indicated by o, x symbols. Scale bars: 10 microns.

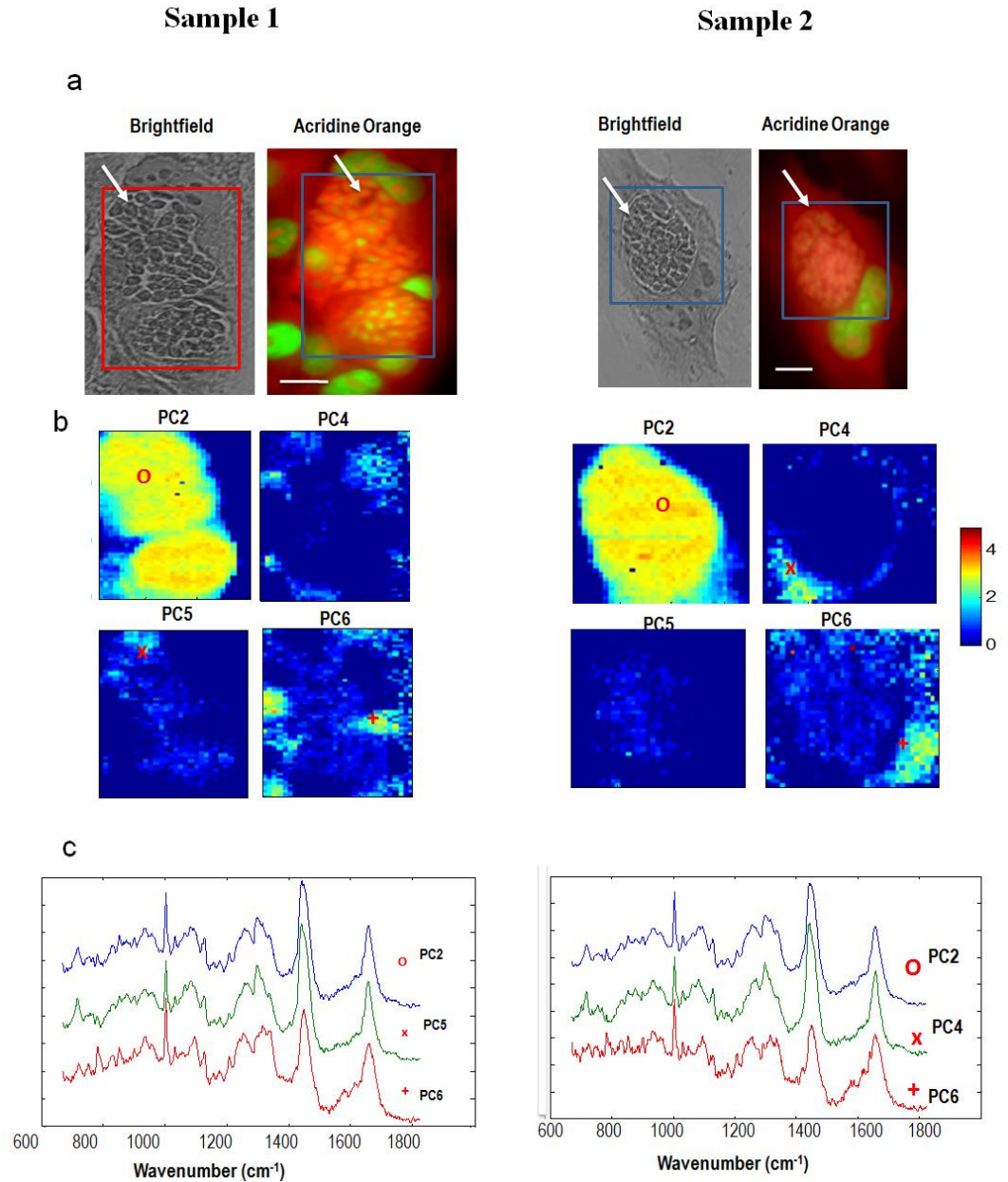


Figure 4.5: Raman analysis of *T. gondii* infected cells at 48 hr PI. (a) Bright field and fluorescence images (recorded after Raman spectroscopy measurements), white arrows represents the position of parasites in the cytoplasm of host cells. (b) Maps corresponding to principal components PC2, PC4, PC5 and PC6. (c) Selected Raman spectra at locations indicated by o, x symbols. Scale bars: 10 microns.

4.3.3 Raman spectroscopy of live *T. gondii* during infection

After establishing the PCA model based on the time-course Raman spectral maps measured from paraformaldehyde-fixed cells, the model was applied to new

Raman spectral datasets measured from live cells. Human retinal cells were cultured in titanium cell-chambers, and after the addition of live *T. gondii* tachyzoites, the cell chambers were placed on the Raman microscope in the environmental enclosure for a period of 48 hr (additional cell-chambers with control healthy retinal cells were also included). Figure 4.6 shows the scores corresponding to PC2, PC4, PC5 and PC6 (calculated using the model developed for the fixed cells) for the live *T. gondii*-infected retinal cells. While the scores for PC4, PC5 and PC6 showed no spectral differences between the control live and fixed cells, the PC2 scores for the live *T. gondii* tachyzoites matched the scores for the fixed *T. gondii* at 48 hr PI. The results agree with the bright field and AO images (Figure 4.7a) that showed the parasite vacuoles contained 6-10 parasites growing in the cytoplasm of the host cells. The PC2 maps show good correlation with the AO images and the selected Raman spectra indicated strong bands corresponding to proteins and lipids.

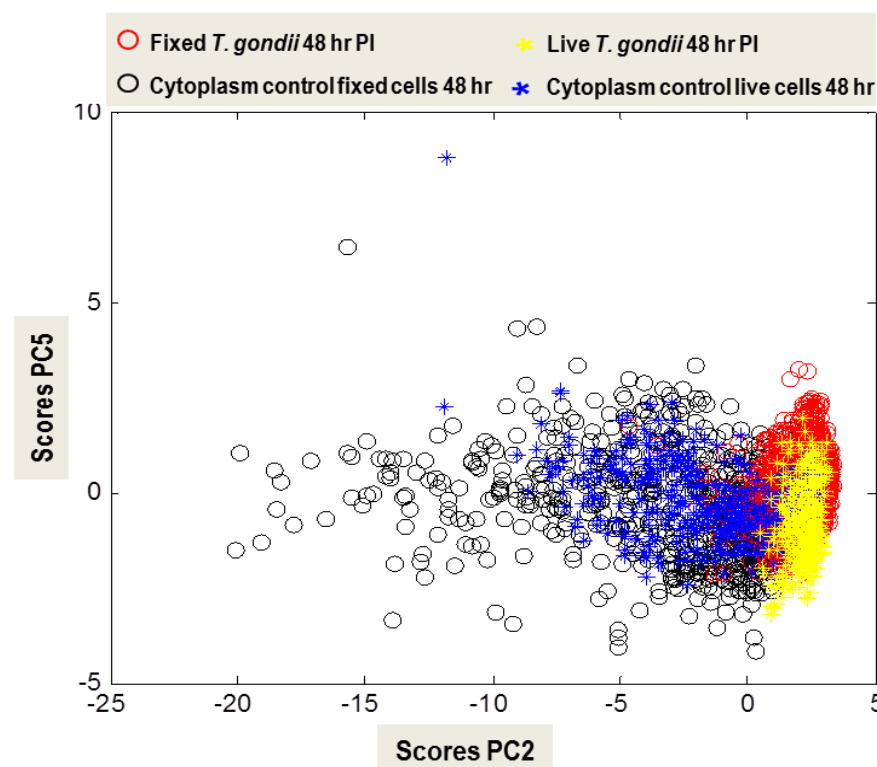


Figure 4.6: PCA analysis of the Raman spectra for live *T. gondii* tachyzoites inside ARPE-19 cells after 48 hr PI. The scores corresponding to PC2 and PC5 were calculated using the model developed for fixed cells (Fig. 4.2).

Lipid droplets, major lipid storage structures, are composed of a triglyceride and cholesteryl ester core with a surrounding monolayer of phospholipid, cholesterol, and a variety of associated proteins with diverse functions in cell metabolism, signaling, and inflammation [5,6]. The association between *T. gondii* infection and increased concentrations of proteins and lipids is consistent with a previous report where the size and number of lipid droplets, whose major component is TGAs, increased in cells infected by *T. gondii* [7]. The interaction of parasite proteins with these lipid bodies is important for the replication of intracellular pathogen, such as *T. gondii* and for the biogenesis of new parasite particles [8]. The intense bands at 788 cm^{-1} also indicated increased amount of nucleic acids, also confirmed by the intense AO fluorescence intensity. The source of the nucleic acids' traces, host or parasite origin, is unknown. However,

the concentration of nucleic acids was found to increase in the infected cultures in proportion to the proliferation of parasite, suggesting the source of the nucleic acids to be of parasite origin. This finding is consistent with a recently reported finding in the related protozoan *N. caninum* [8].

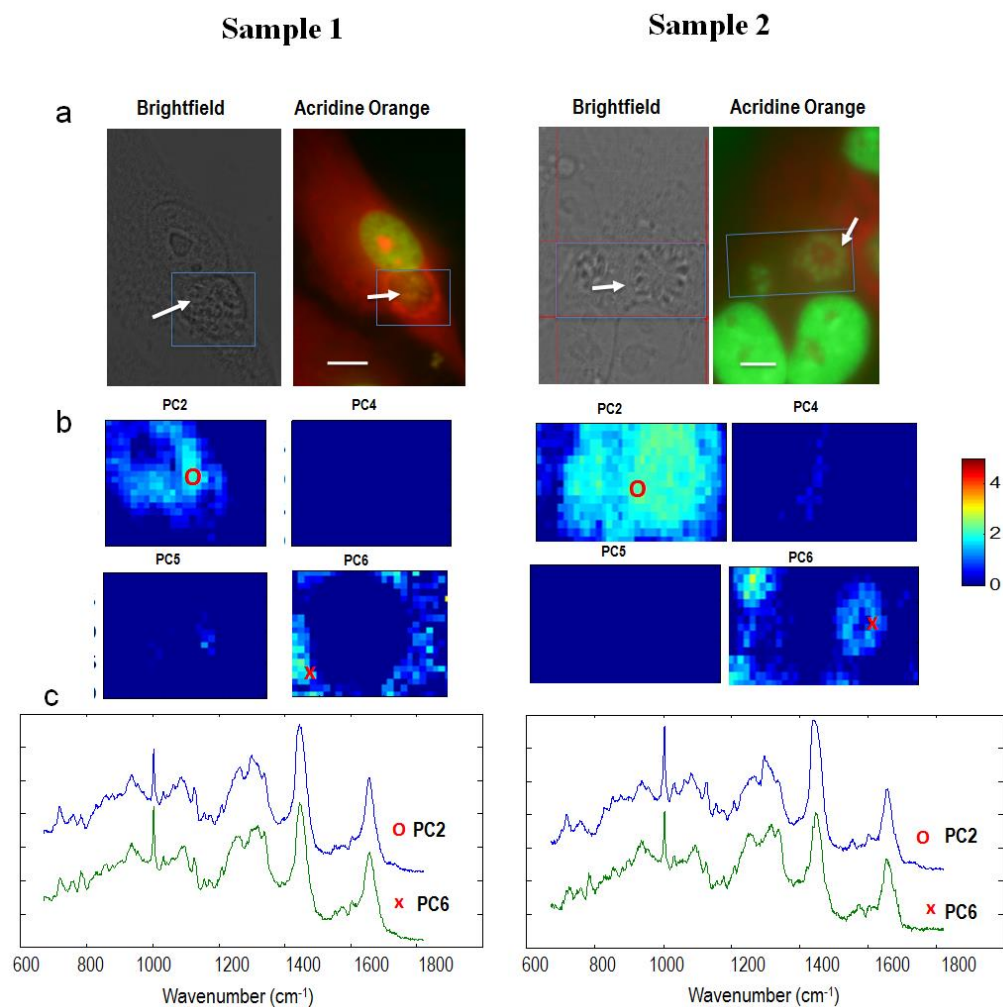


Figure 4.7: Live ARPE-19 cells infected with *T. gondii* at 48 hr PI. (a) Bright field and fluorescence images (recorded after Raman spectroscopy measurements), white arrows represents the position of parasites inside host cell cytoplasm. (b) Maps corresponding to principal components PC2, PC4, PC5 and PC6. (c) Selected Raman spectra at locations indicated by o, x, + symbols. Scale bars: 10 μm.

The application of RMS in studying host-pathogen interaction in the field of parasitology is promising. Raman spectroscopy was successfully used to

compare healthy erythrocytes and erythrocytes infected with *Plasmodium falciparum* [9]. RMS was also used to monitor molecular changes in red blood cells during *Plasmodium* infection in mice, following malaria disease progression over the course of 7 days [10]. Changes in the Raman spectra of cells was associated with the loss of hemoglobin and increase of hemozoin 4 days PI [10]. The use of ARPE-19 cells is relevant and has medical importance because this cell line can be used as a model of blood-retinal barrier, which is fundamentally important for the protection of the eye and for maintenance of vital ocular functions.

4.4 Conclusions

Raman micro-spectroscopy was used to measure label-free molecular properties of *T. gondii*- infected host cells. Significant increase in protein and lipid concentration was detected associated with development of infection as confirmed by time-course experiments on both paraformaldehyde-fixed and live infected cells. The development of *T. gondii* tachyzoites was also accompanied by an increase in nucleic acids as early as 6 hr PI. While this study demonstrates the potential of Raman spectroscopy to detect molecular changes during host–pathogen interaction, the current method does not provide sufficient specificity to pinpoint the main proteins or lipids that are affected during this biological process. Further studies have been done using isotope labelled molecules to improve the specificity and discrimination between biomolecules originating from the *T. gondii* and host cells. The experiments given in this chapter are likely to provide further information related to molecular exchange between pathogen and host cells. Understanding the time- and spatially-dependent molecular

interactions between pathogens and host cells may provide a useful platform for screening compounds with potential neuro-protective activity for eminent effects of changes in brain infection control practices.

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Chapter 5

Tracing amino acid exchange during host-pathogen interaction by combined stable-isotope time-resolved Raman spectral imaging

The objective of this study was to investigate the temporal and spatial interchange of the aromatic amino acid phenylalanine (Phe) between human retinal pigment epithelial cell line (ARPE-19) and tachyzoites of the apicomplexan protozoan parasite *T. gondii*.

5.1 Introduction

T. gondii is the most prevalent protozoal infection of the Central Nervous System. It is the causative agent of a spectrum of human and animal diseases ranging from abortion to neurological disorders [1]. This obligatory intracellular apicomplexan protozoan parasite has the capacity to infect all vertebrate species and survives within its own membrane-bound compartment referred to as the parasitophorous vacuole (PV) within virtually any phagocytic and nonphagocytic cell type [2]. The broad range of cells amenable to infection by *T. gondii* reflects the plasticity of the parasite's metabolism and versatility in scavenging nutrients to support its intracellular growth [13,4]. Nutrient acquisition from the host is a strategic utilization of essential nutrients, such as

amino acids (AA), lipids and glucose to support the parasite's metabolic requirements. *T. gondii* and other apicomplexans are fast-replicating organisms, and thus are critically dependent on host-cell nutrients as carbon and energy sources for the extensive membrane biogenesis imposed during their replication. For example, *T. gondii* takes up non-essential amino acids, such as serine for incorporation into lipids [5].

Even though *T. gondii* can synthesise some AA *de novo* this parasite lacks synthetic pathways for some AA, which must be retrieved from the host cells [6-9]. Therefore, the pools of amino acids in the host cytosol constitute an important reservoir to complement the endogenous production of amino acids in the parasite. For example, the replication of *T. gondii* is proportional to the concentration of tryptophan in the host cell [10,11]. Another example is Phenylalanine (Phe), which is one of the most vital AA needed by both mammalian cells and *T. gondii*. *T. gondii* genome encodes hydroxylase enzymes, which can metabolize both Phe and Tyrosin (Tyr) to L-DOPA, with a substrate preference for Tyr. [12]. Although the pathway of Phe synthesis has been studied little is known regarding the transport of Phe from mammalian cell to the parasite and the formation of precursor pool, as well as protein synthesis and turnover within the parasite. Unravelling these complex processes requires novel approaches that are capable of monitoring the shuttling of AA from or toward the host during *T. gondii* infection.

Isotopically labelled AA tracer is frequently used to trace the kinetics of incorporation of a labelled AA into newly synthesized proteins, thus can be

effectively used to characterize the acquisition of AA by the growing parasite. Various isotope labelling approaches exist for studying protein dynamics and turnover, including incorporation of a stable isotope label via either metabolic incorporation in cell culture using stable isotope labelling by amino acids in cell culture (SILAC), chemical derivatization (GC-MS, ICAT, iTRAQ, TMT), or enzymatically catalyzed incorporation (^{18}O labelling). However, these approaches are invasive, cost- and/or time-prohibitive, or might not be amenable to the biological system of interest. Also, these methods cannot elucidate the kinetic events that occur rapidly and change from cell to cell, and thus cannot describe the complex dynamic interactions between engaging host and parasite systems in a quantitative manner.

Raman micro-spectroscopy (RMS) is a non-invasive, label-free imaging technique that can enable non-invasive molecular analysis of dynamic events in live cells *in-vitro* [13]. Its utility has been confirmed in studies covering a wide spectrum of applications, including, cellular differentiation [14-16], apoptosis [17-19], drug-cell interactions [20]. Also, we have demonstrated the ability of RMS to detect molecular changes during parasite development within host cells [21]. However, this approach could not provide enough specificity to reveal which molecules (e.g., nucleotides, glucose, lipids or amino acids) are altered during host-*T. gondii* interaction.

Combining stable-isotope labelling with RMS can increase the chemical specificity of Raman bands, and identify the origin of the molecular species and monitor its metabolism [22-26]. Metabolic labelling with stable-isotope has been

a prominent technique for examining the lipid uptake and metabolism [27]. In this study, we show for the first time that it is possible to combine time-resolved RMS and stable-isotope labelling approach to monitor the exchange of molecules between individual cells in real-time. Here, we monitor the kinetic of labelled Phe acquisition by *T. gondii* from the host cell during the first 48 h of the parasite's intracellular development. Identification of parasite metabolic needs during infection provides insight into the nutritional cues being sensed by the parasite and can reveal unique targets for novel therapeutics.

5.2 Methods

5.2.1 Reagents: L-Phenylalanine (D8, 98%) (Cat no. DLM-372-1) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA) whereas L-Phenylalanine (Cat no. P5482), paraformaldehyde, trypan blue, human serum albumin, and acridine orange were purchased from Sigma Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium low glucose, pyruvate (DMEM, Cat no. 11885) was purchased from Life technologies, Grand Island, NY. Fetal bovine serum (FBS) and penicillin/streptomycin were obtained from Invitrogen Corp., Carlsbad, CA.

5.2.2 Cell Culture, Growth and preparation of parasites and *In vitro* infection protocol. These are the same as explained in Chapter4, sections 4.2.1, 4.2.2, 4.2.3.

5.2.3 Cell doubling time. ARPE-19 cells were trypsinized in the exponential growth phase. Equal numbers of cells ($\sim 1 \times 10^4$) were plated in 6-well culture

plates (day 0) and cultured in 5% CO₂ at 37°C. Cell proliferation was quantified every 24 h for 6 days in order to establish the growth profile of the culture. Cells were counted by using hemocytometer and disposable cell counting chamber. This experiment was conducted in triplicate.

5.2.4 Stable isotope labelling by amino acids (SILAC). The aim of these experiments was to determine the lowest concentration of the labelled AA that provides the best Raman spectral signal shift. Amino acid-type selective labeling was achieved by formulating a medium containing all amino acids in unlabeled form, but Phe, which was added with the desired isotope label. To minimize the background signal of the L-Phe ARPE-19 cells were synchronized overnight in serum-deficient, Phe-free customised DMEM culture medium. The cells were subsequently incubated with SILAC DMEM medium supplemented with various concentrations of Phe(D8). Cells were subjected to Raman imaging analysis daily to determine the time taken by L-Phe(D8) to substitute L-Phe within host cells. The concentration of 66mg/500ml of L-Phe(D8) (i.e. twice the concentration of L-Phe present in the normal media) was found to be the lowest concentration that showed a shift.

5.2.5 Infection of host cells with *T. gondii*. ARPE-19 cells (1×10^4 cells/ml) were seeded in titanium cell-chambers, which are fitted in 6-well plastic cell culture plates, with a volume of 2 ml DMEM/chamber. The titanium cell chambers were purpose-built to enable acquisition of Raman spectra of the cells and incorporated MgF₂ coverslips (0.17 mm thick) at the bottom. Cells were allowed to grow overnight by incubation at 37°C in a humidified atmosphere of

5% CO₂ – 95% air. Then, cells were serum starved for 18 h, followed by maintaining in L-Phe(D8)-supplemented DMEM for a minimum of 5 doubling times to ensure that cells are uniformly labelled and that L-Phe is completely substituted by L-Phe(D8). Before introducing the parasites to cells the culture medium was replaced with L-Phe-free media to avoid any false labelling of the parasite with L-Phe(D8) while in the extracellular medium before cell invasion. Parasites are grown in a medium containing L-Phe, but they were transferred to L-Phe-free media before adding them to the cells and during host cell invasion to avoid introducing L-Phe to L-Phe(D8)-labelled cells and to ensure that the only source of Phe to the parasites comes from the host cells. Infection was initiated by adding *T. gondii* tachyzoites at a multiplicity of infection (MOI) of 1 cell to 2 parasites (1C:2P) in 2-ml fresh L-Phe(D8)-free DMEM. The control chambers received only 2-ml DMEM (mock-infected). Culture plates were then incubated to allow infection to progress within host cells. Parasite and control cultures were sampled at 0, 6, 24 and 48 h post infection (PI). At each sampling time two chambers (one infected and one control) were collected from the 6-well culture plate and subjected to Raman Spectroscopy imaging. At least 10 infected cells were analyzed per chamber. In a separate experiment we incubated free extracellular tachyzoites that are recovered from L-Phe culture media in D-Phe media. These parasites failed to take L-Phe(D8) from media even after 24 h.

5.2.6 Time course Raman imaging. A 785 nm ~120 mW laser (before objective) (Ti:sapphire laser, Spectra-Physics) was used for excitation of Raman spectra. Raman spectral imaging was performed by scanning the cells through the laser focus in a raster pattern (1 µm step size) acquiring Raman spectra at

each position (1s/pixel). The schematic diagram and description of Raman micro-spectrometer system is shown in Figure 3.1 in chapter 3.

5.2.7 Fluorescence imaging

AO staining and fluorescence imaging of Healthy and *T. gondii*-infected cells were performed, on the same cells, after completion of the Raman spectral imaging. The method of the staining is explained in Chapter 3, section 3.4.2.

5.3 Image analysis and data processing. Data pre-processing and analysis was done by using in-house built functions in Matlab (The MathWorks, Natick, MA). The spectra are collected separately from host cell cytoplasm, nucleus and the parasitophorous vacuole, which encloses the growing parasites within the cytoplasm of infected host cell. Data pre-processing involved cosmic ray removal and background subtraction. Noise reduction was performed by using singular value decomposition. The height of the peak was measured after a local linear baseline was subtracted. Human serum albumin (CAS number 70024-90-7, Sigma Aldrich, UK) was used for the calibration of the Raman spectrometer (30 Phe residues per albumin molecule).

5.4 Results

5.4.1 Host cell uptake of L-Phe(D8). First, we investigated the rate of substitution of L-Phe by L-Phe(D8) tracer within the host cells. ARPE-19 cells were metabolically labelled by adding L-Phe(D8) to the Phe-free customised DMEM medium. Phe is an essential amino acid and the only source of Phe for the cells is the culture medium. The uptake of L-Phe(D8) is expected to be driven

mostly by new protein biosynthesis during cell division as well as during protein turnover in non-dividing cells. The doubling time (DT) of ARPE-19 cells grown *in vitro* was determined from a series of 7 time points collected over a period of 7 days by using cell counting, revealing a DT of 24 h. To achieve complete substitution of L-Phe(D8), ARPE-19 cells were maintained in serum-free DMEM overnight; then, cells were maintained in complete DMEM with serum, but free of any Phe for 24 h. Then, culture medium was replaced by L-Phe(D8)-supplemented medium. Figures 1 show average Raman spectra measured from the cytoplasm and nucleus of ARPE-19 cells growing in L-Phe(D8)-containing medium daily for 7 days. Prior to supplementing the culture medium with L-Phe(D8), the sharp Raman band corresponding to the ring-breathing mode of L-Phe was identified at 1004 cm^{-1} . Upon using culture medium containing L-Phe(D8) the frequency of this vibrational mode shifts to 960 cm^{-1} . Fig. 5.1 clearly shows the time-dependent increase in the intensity of the 960 cm^{-1} Raman band corresponding to L-Phe(D8), concomitantly with a decrease in the intensity of the 1004 cm^{-1} band, which corresponds to L-Phe. After one doubling time (1 day), the ratio $I_{960}/(I_{960}+I_{1004})$ is ~ 0.53 for the spectra measured in the cytoplasm and ~ 0.60 in the nucleus, indicating a similar distribution of newly synthesized proteins in the host cells. The substitution increases with time, the ratio $I_{960}/(I_{960}+I_{1004})$ becomes ~ 0.72 at 4 days, both in the cytoplasm and nucleus, and reached 0.9-1.0 at days 5 when the Raman band at 1004 cm^{-1} cannot be detected.

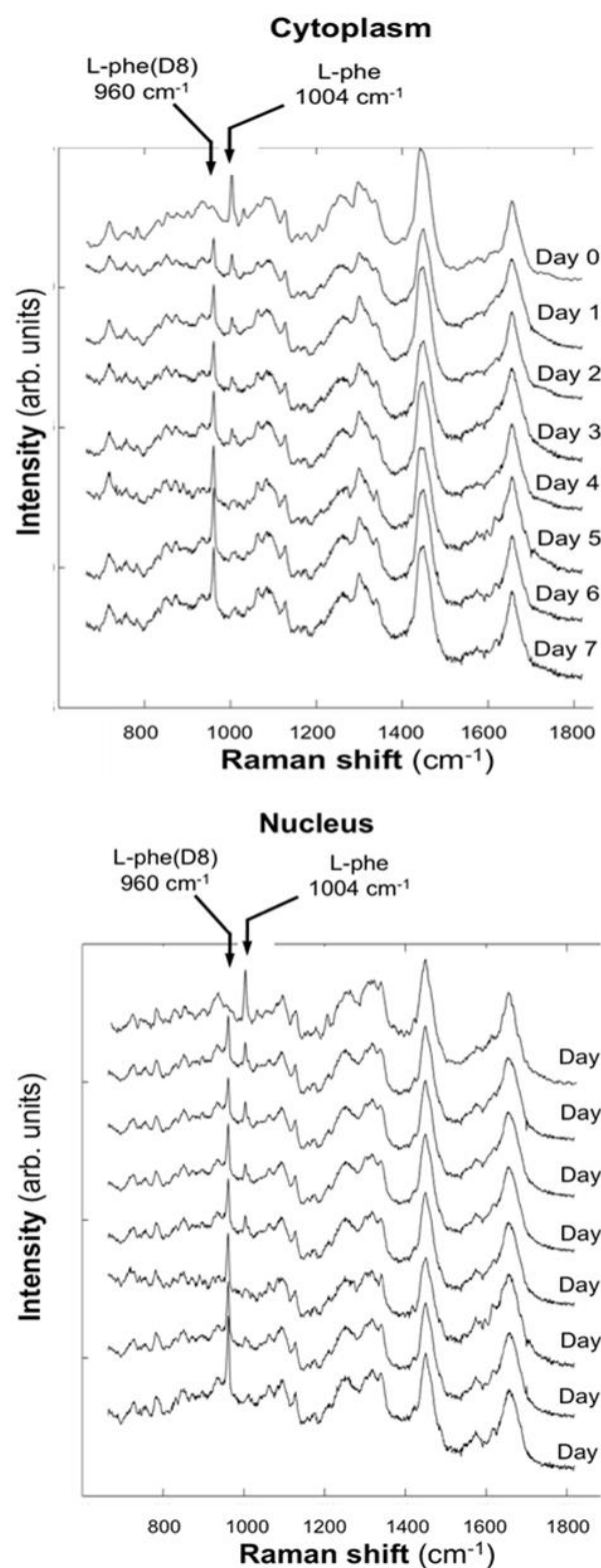


Figure 5.1: Time course for L-Phe(D8) uptake by growing ARPE-19 cells. Raman spectra measured in the cytoplasm and nucleus of ARPE-19 cells cultured in L-Phe(D8)-containing medium at the indicated time points. Spectra are shifted vertically for clarity.

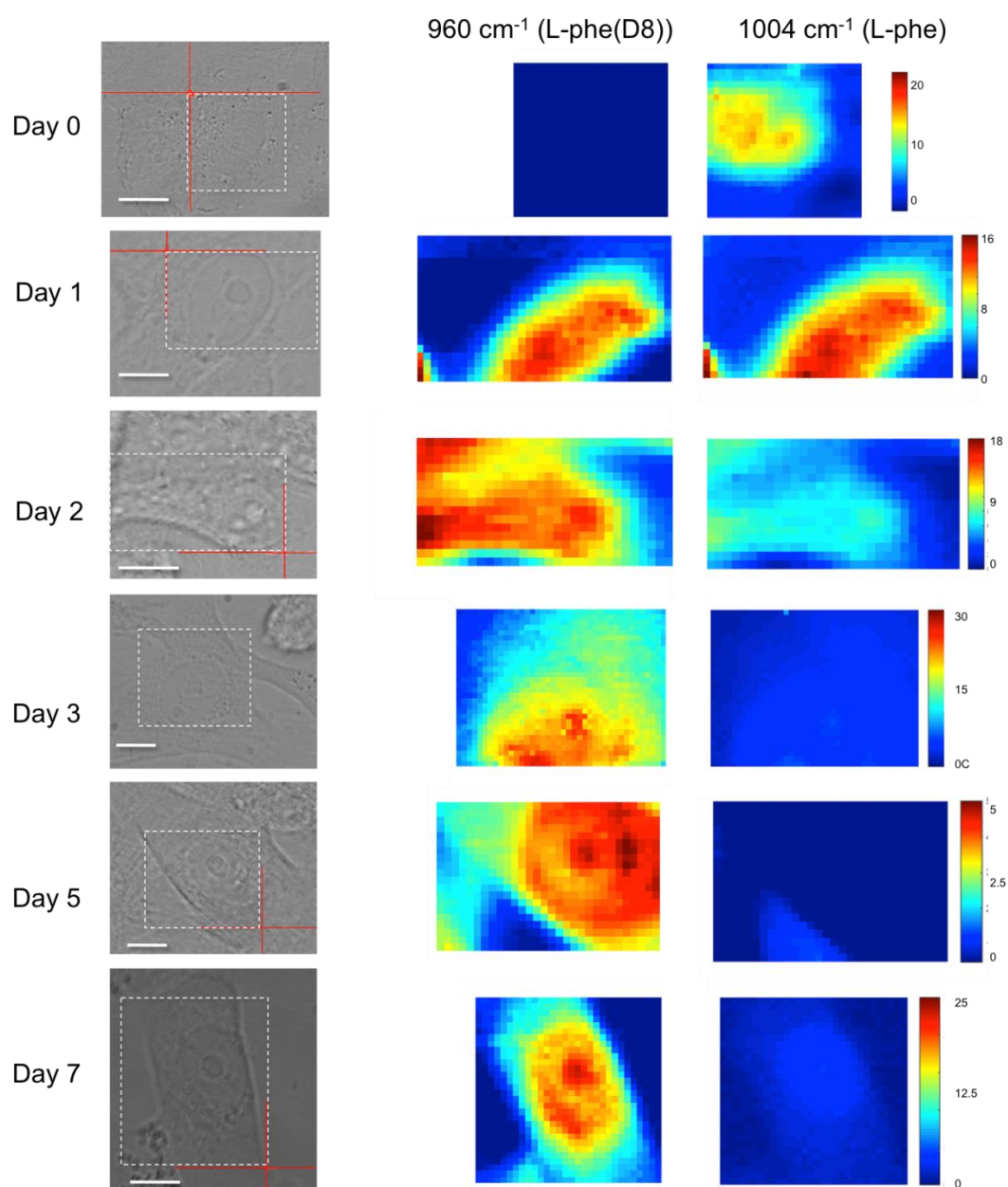


Figure 5.2: Time course accumulation ratio of L-Phe(D8) (960 cm^{-1}) in ARPE-19 cells. Raman spectral images corresponding to L-Phe (1004 cm^{-1}) and L-Phe(D8) (960 cm^{-1}) for ARPE-19 cells grown in L-Phe(D8)-supplemented medium. Scale bars: $10\text{ }\mu\text{m}$.

Raman micro-spectroscopy was also used to evaluate the intracellular distribution and accumulation of L-Phe(D8) over time course (Fig. 5.2). At short incubation times in L-Phe(D8) medium, the Raman spectral maps corresponding to the 1004 cm^{-1} and 960 cm^{-1} bands indicate similar patterns for the distribution

of L-Phe and L-Phe(D8), supporting their biological equivalence. Comparison between the Raman spectra recorded from locations in the cell cytoplasm and nucleus indicates that the incorporation of L-Phe(D8) occurs at the similar rates.

While the 1004 cm^{-1} and 960 cm^{-1} Raman bands are assigned to ring-breathing vibrations of L-Phe and L-Phe(D8), respectively, the intensity of the 1660 cm^{-1} Raman band corresponds to the Amide I vibrational modes and can be related to the overall level of protein synthesis. Thus, the ratio between the band intensities of the ring-breathing modes of Phe ($I_{960}+I_{1004}$) and the Amide I (I_{1660}) can be linked to the level of incorporation of Phe into proteins. Fig. 5.3a presents the calculated values of the ratio $(I_{960}+I_{1004})/I_{1660}$ for cells grown *in-vitro* in culture media containing L-Phe and L-Phe(D8) at different time points. It is interesting to observe that regardless of the incubation time, the uptaken L-Phe-(D8) is incorporated into proteins at similar levels as L-Phe under normal culture conditions.

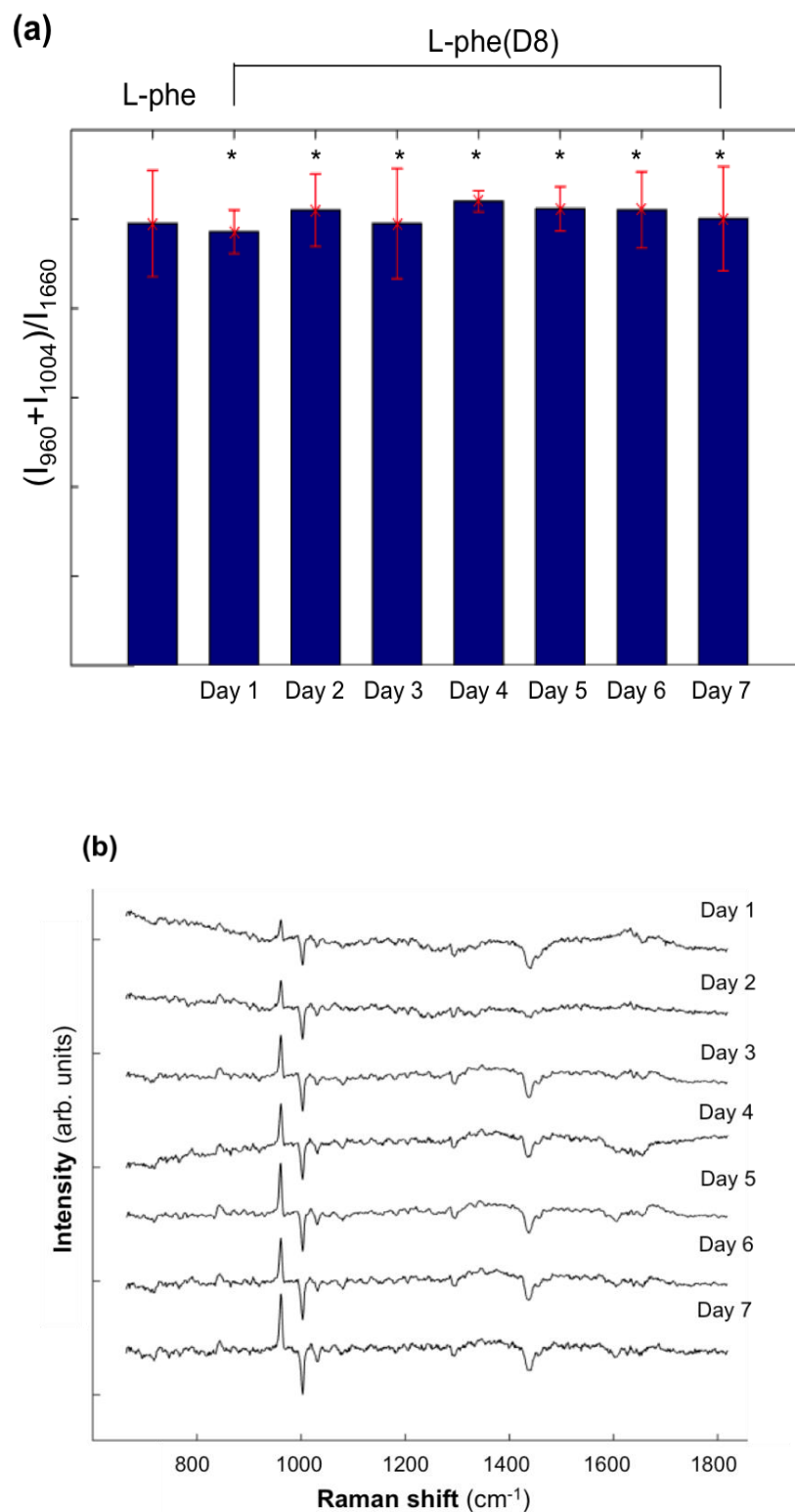


Figure 5.3: Time course of L-Phe(D8) uptake. (a) Calculated ratio $(I_{1004} + I_{960})/I_{1660}$ for ARPE-19 cells grown in L-Phe and L-Phe(D8 (marked by *)) culture media. (b) Computed difference Raman spectra acquired from cells cultured in L-Phe and L-Phe(D8) daily for 7 days. Spectra are shifted vertically for clarity.

To determine whether the uptaken L-Phe(D8) is also metabolized by the ARPE-19 cells, the difference between the Raman spectra for cells cultured in medium containing L-Phe and L-Phe(D8) were calculated (Fig. 5.3a). The results indicate that apart from the downshift of the ring-breathing mode of Phe from 1004 cm^{-1} to 960 cm^{-1} , no other major changes can be observed that could be related to the isotope substitution. Of particular interest is tyrosine (Tyr), a metabolic product of Phe. Typically, in the Raman spectra of cells, Tyr can be identified as a spectral doublet at 828 cm^{-1} and 854 cm^{-1} , which corresponds to a Fermi resonance between the ring-breathing mode and the overtone of the out-of-plane ring-bending mode. Upon substitution of H with D in Tyr, the frequency of the out-of-plane ring-bending mode was found to drop to 362 cm^{-1} (overtone 724 cm^{-1}) while the ring-breathing mode occurs at $\sim 791\text{ cm}^{-1}$. Because of the difference in the frequencies for these two vibrational modes, the Fermi resonance is removed and only a single peak at $\sim 791\text{ cm}^{-1}$ was observed around in the Raman spectrum of Tyr(D4). As the calculated difference spectra in Fig. 5.3a show no changes in the Raman bands in the $770\text{-}790\text{ cm}^{-1}$, but rather an increased peak at $\sim 840\text{ cm}^{-1}$ it can be assumed that no conversion of L-Phe(D8) into Tyr(D7) can be detected. The results indicate that the L-Phe(D8) is directly incorporated into newly synthesized protein. It is also important to mention that the detection of the 791 cm^{-1} band of Tyr(D7) may be hindered by the overlap with the Raman bands corresponding to nucleic acids (788 cm^{-1} O-P-O phosphodiester stretching vibrations).

5.4.2 Differential uptake of L-Phe(D8) by extra-cellular versus intracellular *T. gondii* after. We know that *T. gondii* tachyzoites while extracellular are incapable of replication. But, we do not know whether *T. gondii* tachyzoites outside mammalian cells are able to acquire Phe directly from the culture media. Hence, it was important to investigate whether the information provided by the Raman spectra reflects solely molecular exchange between *T. gondii* and host cells and that there is no contribution from the culture medium, which also contains L-Phe(D8). Fig. 5.4 compares the Raman spectra for *T. gondii* infecting ARPE-19 cells and *T. gondii* tachyzoites that were still extracellular and have not invaded the host ARPE-19 cells yet. At 9h PI, individual *T. gondii* tachyzoites were observed inside ARPE-19 cells and the Raman spectra indicated a high intensity band at 960cm^{-1} assigned to L-Phe(D8), while the corresponding band at 1004 cm^{-1} corresponds to L-Phe was ~4-fold lower. At 12 h the 1004cm^{-1} band cannot be detected in the spectra of *T. gondii*. These results indicate a very rapid uptake of L-Phe(D8) by *T. gondii* even before the start of the parasite division which occurs approximately around 12 h. In contrast, the Raman spectra of *T. gondii* that did not enter the ARPE-19 cells (marked by * in Fig. 5.4), contain intense Raman bands at 1004 cm^{-1} corresponding to L-Phe and only small peaks (~5-fold lower) at 960 cm^{-1} , both at 9 h and 12. The fact that intensities of neither the 1004 cm^{-1} nor 960 cm^{-1} bands change from 9 h PI to 12 h PI indicates that this peak is the contribution of L-Phe(D8) molecules in the underlying ARPE-19 cell. These results indicate that the *T. gondii* cannot uptake Phe directly from the culture medium and that the Raman bands at 960cm^{-1} identified in the intracellular *T. gondii* (marked by o in Fig. 5.4) originate from L-Phe(D8) acquired only from the host ARPE-19

cells after infection. The images recorded at 48 h PI also show that *T. gondii* developed and replicated successfully as the PV contained many tachyzoites at 48 h. No differences were observed in the growth of *T. gondii* infecting ARPE-19 cells cultured in L-Phe or L-Phe(D8). These results show that the substitution of L-Phe with L-Phe(D8) and the exposure of the *T. gondii* and ARPE-19 cells to 785 nm laser during the Raman spectral measurements did not adversely affect the viability of the cells.

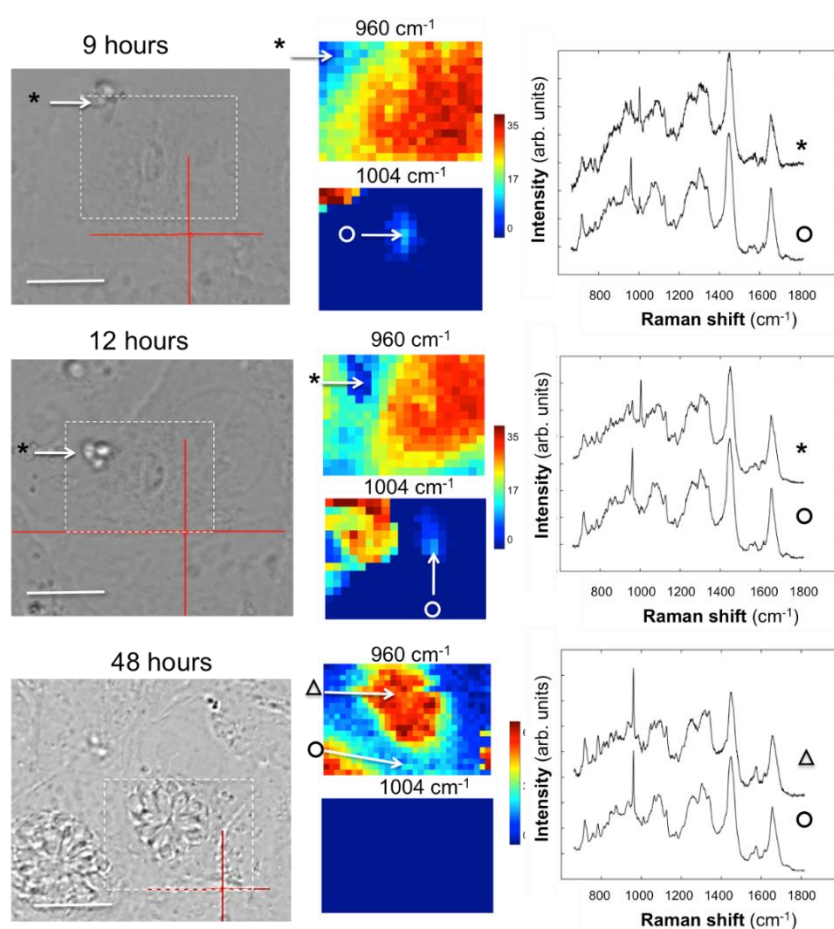


Figure 5.4: Comparison between Raman spectra of *T. gondii* tachyzoites within host cells and those that did not enter host cells and remain extracellular. (top) 9 h: Raman spectra of parasites in and out host cells; (middle) 24 h Raman spectra show that all proteins in parasite have L-Phe(D8), normal L-Phe can no longer be detected (parasites start to divide); (bottom) at 48 h, note the large parasitophorous vacuole full of parasites. All spectra are shifted vertically for clarity. Scale bars: 10 μ m.

5.4.3 Time- and spatially-resolved exchange of proteins between host and pathogen. To obtain detailed information regarding metabolism and exchange of Phe between the host ARPE-19 cells and *T. gondii*, time-resolved Raman spectral mapping of *T. gondii* was performed from 1h to 48h PI. Figure 5.5a and Figure 5.5b presents the time resolved Raman spectra and spectral maps corresponding to the 960 cm^{-1} and 1004 cm^{-1} of four live *T. gondii* tachyzoites during the invasion of ARPE-19 cells. Intense Raman bands corresponding to L-Phe(D8) at 960 cm^{-1} can be observed at as early as 2 h PI, concomitant with a decrease in the intensity of the 1004 cm^{-1} assigned to L-Phe. It is also interesting to note that Raman bands at 1004 cm^{-1} can be detected in the cytoplasm of the APRE-19 cells, suggesting release of proteins by *T. gondii* into the host. The intensity of the 960 cm^{-1} band increases with time, indicating a rapid uptake of L-Phe(D8) by *T. gondii*. By 9-12h PI, the Raman band at 1004 cm^{-1} is no longer detected in the Raman spectra of *T. gondii*, suggesting that the initial L-Phe in *T. gondii* is completely substituted with L-Phe(D8) acquired from the host cells. Although during this time the *T. gondii* grow in size, they divided only at ~12h PI, by then L-Phe has been replaced by L-Phe(D8).

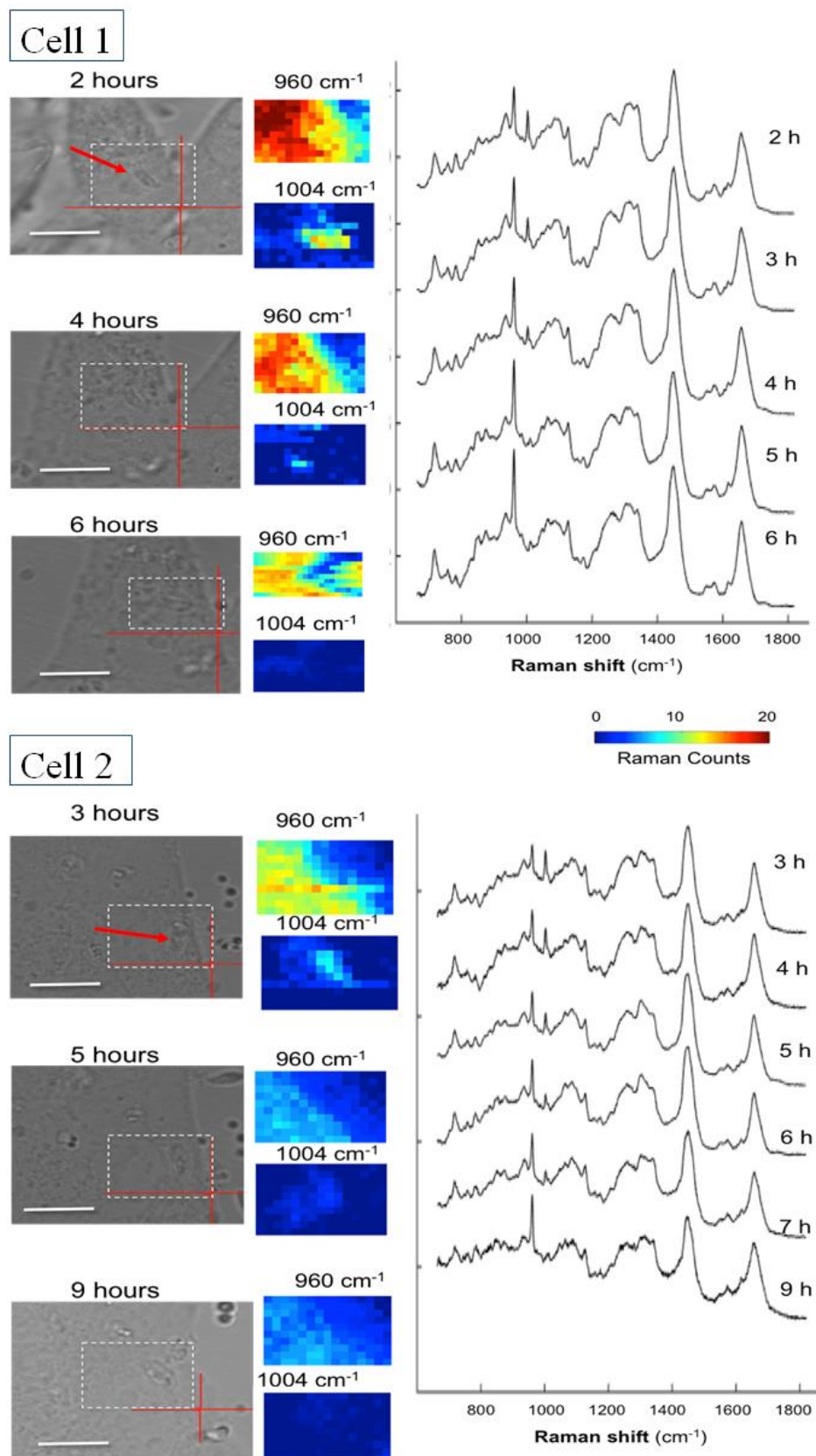
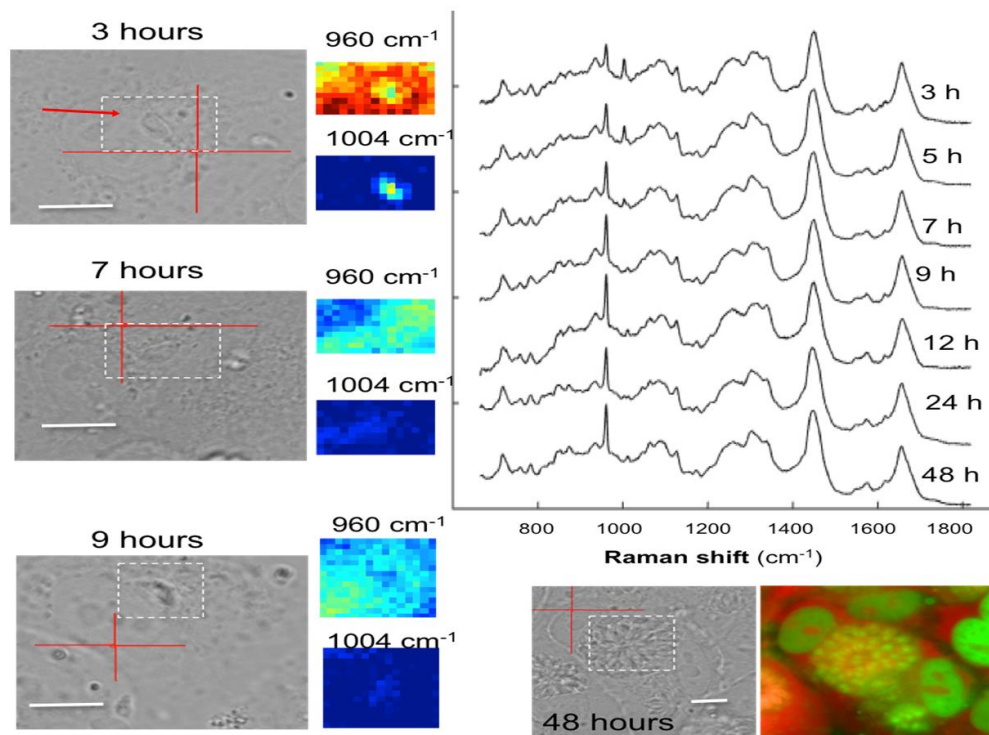


Figure 5.5 (a) cell 1 and cell 2: Typical time-course Raman spectra and maps for the 1004 cm^{-1} (L-Phe) and 960 cm^{-1} (L-Phe(D8)) bands of live *T. gondii* infecting ARPE-1 cells. The measured areas are indicated by the white dashed

rectangles; red arrows show the position of parasites inside host cell cytoplasm. Acridine orange (AO) staining was carried out at 48 h after infection. All spectra are shifted vertically for clarity. *Scale bars: 10 μm .*

Cell 3



Cell 4

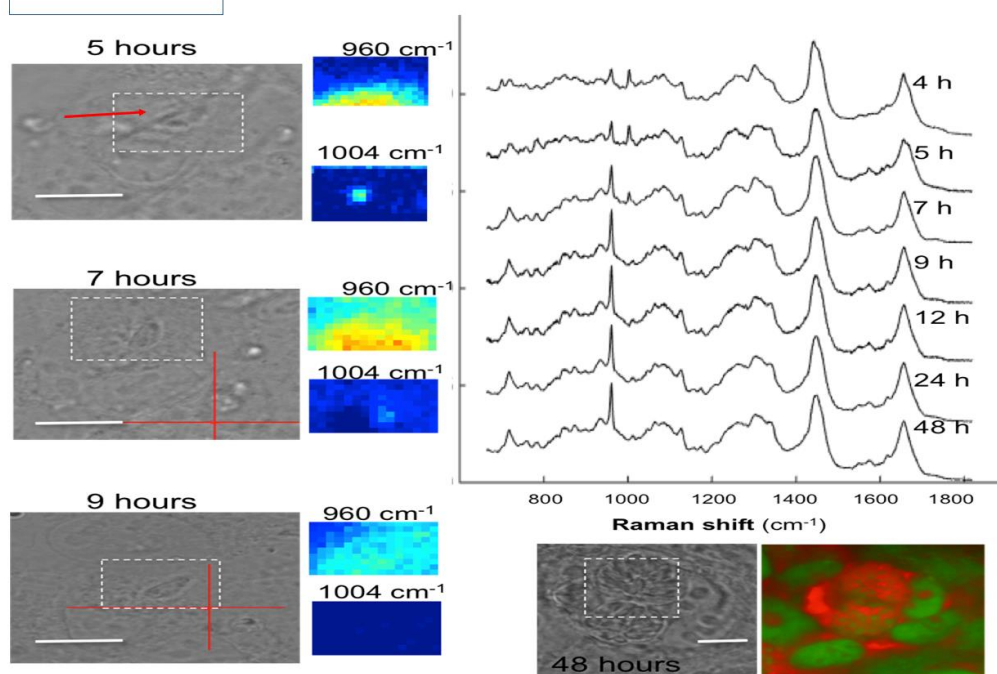


Figure 5.5 (b) cell 3 and cell 4. Typical time-course Raman spectra and maps for the 1004 cm^{-1} (L-Phe) and 960 cm^{-1} (L-Phe(D8)) bands of live *T. gondii* infecting ARPE-1 cells. The measured areas are indicated by the white dashed

rectangles; red arrows show the position of parasites inside host cell cytoplasm. Acridine orange (AO) staining was carried out at 48 h after infection. All spectra are shifted vertically for clarity. *Scale bars*: 10 μm .

5.4.4 Dynamic of uptake of L-Phe(D8) at single parasite level. Fig. 5.6 presents the kinetics of Phe exchange between *T. gondii* and the host ARPE-1 cells. The uptake was expressed as the percentage of L-Phe(D8) from the total Phe calculated as the ratio $\text{I}_{960}/(\text{I}_{960}+\text{I}_{1004})$. Compared to other techniques that require large populations of cells and provide only average results, Raman microscopy can measure the rates of Phe transport for individual live *T. gondii*. Figure 5.6 shows that more than 90% of *T. gondii* have a rapid uptake of Phe from the host cells, as the L-Phe(D8) reached ~50% at 2-3 h PI. It is interesting to note that after ~3 h PI, two trends can be observed. For the majority of *T. gondii* the transport rate seems to decrease slightly and the complete substitution of L-Phe with L-Phe(D8) is typically observed at 9-12 h. For a smaller group of *T. gondii*, the transport rates remain constant and the saturation with L-Phe(D8) is reached as early as 6 h, which is approximately half the time required for the *T. gondii* to divide.

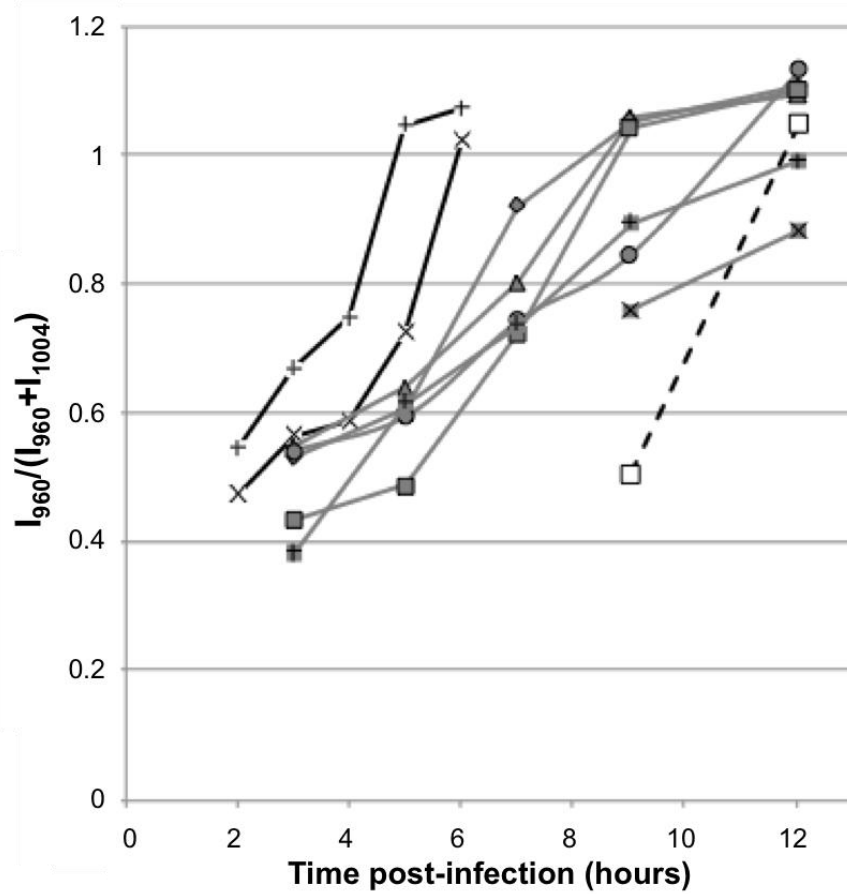


Figure 5.6: Time-resolved uptake of L-Phe(D8) by individual *T. gondii* tachyzoites. Various individual tachyzoites were monitored from the moment they invade host cell. L-Phe(D8) uptake by the tachyzoites was measured every h for 12 hr.

To quantify the transport rates of L-Phe to *T. gondii*, a calibration model was built for the Raman micro-spectrometer based on human serum albumin solution solution in a sample cell with known thickness (Fig. 5.7a). The intensity of the 1004 cm^{-1} Raman band I_{1004} assigned to L-Phe is directly proportional to the number of L-Phe molecules N_{Phe} irradiated by the sample: $I_{1004} = k \times N_{Phe}$. For a 50 mg/ml of albumin solution within the cylindrical laser volume (height 6 μm and $\sim 2 \mu\text{m}^2$ area), the number of L-Phe residues is $\sim 1.6 \times 10^8$. Based on the

intensity of the 1004 cm^{-1} Raman band for the calibration samples (Fig. 5.7a), the calibration constant was calculated to be $k=1.22\times 10^{-7}$ counts/molec.

Although it was not possible to measure the exact height of the cells used in the time-course experiment described in Figs. 5.5 and 5.6, the size of *T. gondii* was estimated to be $\sim 2\text{ }\mu\text{m}$ thickness and $8\text{ }\mu\text{m}$ length based on the bright field microscopy images. Using the height of the 1004 cm^{-1} band in the measured Raman spectra for isolated *T. gondii* (Fig. 5.7b) and an estimated height of $\sim 2\text{ }\mu\text{m}$, the concentration of L-Phe molecules in *T. gondii* was estimated to be $10\pm 4\times 10^6$ molecules/ μm^3 . For a typical value of $\sim 8\text{ }\mu\text{m}$ for the length of *T. gondii*, the total number of Phe molecules in a *T. gondii* was estimated to be $2.6\pm 1\times 10^8$. Based on the results in Fig. 5.6 indicating that all L-Phe are replaced by L-Phe(D) within 6-9 hours after infection, the transport rate of L-Phe(D8) from the host cells to the *T. gondii* is $0.5\text{-}1.6\times 10^4$ molecules/s.

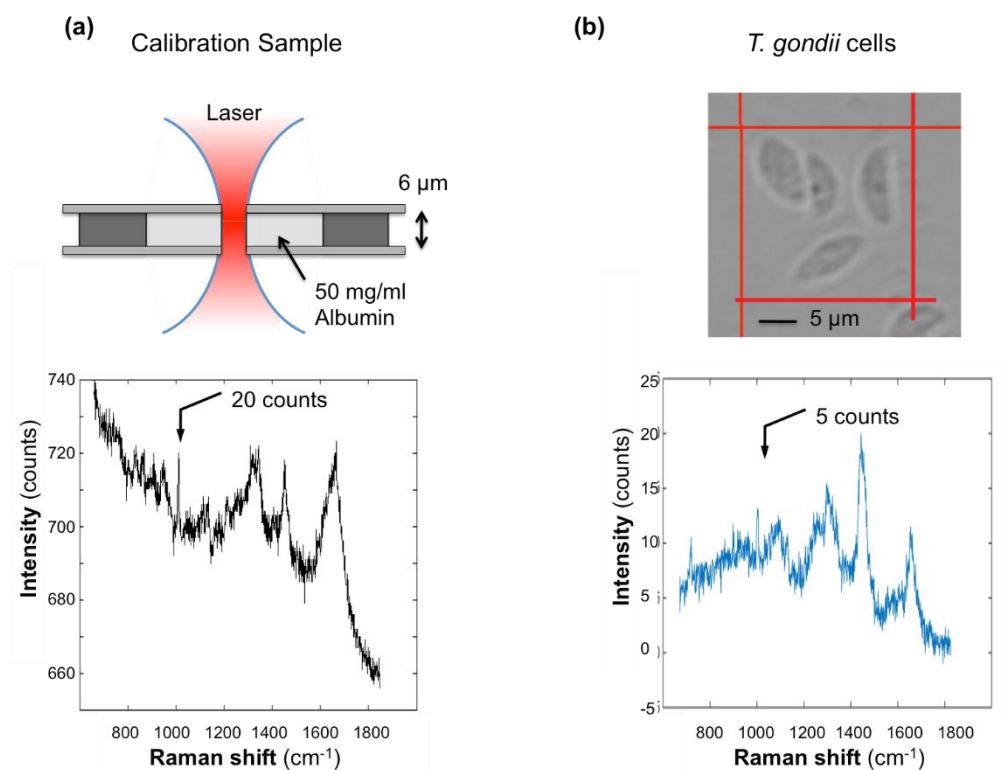


Figure 5.7: Quantification model for L-Phe(D8) in ARPE-19 cells and *T. gondii* based on Raman micro-spectroscopy and AFM. (a) Schematic of the calibration sample of 50 mg/ml human serum albumin in water (thickness 6 μm) and the corresponding Raman spectrum. (b) Images of isolated *T. gondii* tachyzoites and typical Raman spectrum.

5.5 Discussion

Even though Phe is a vital component of proteins in all living organisms the sources of this aromatic amino acid are organism-dependent. For example, mammalian cells are auxotrophic for Phe and must salvage from plants. *In silico* analysis of the nutritional requirements of *T. gondii* has revealed that this parasite is auxotrophic for three AAs, namely Arginine, Histidine, Tryptophan, but can produce 9 AAs (Alanine, Asparagine, Aspartate, Cysteine, Glutamate, Glutamine, Proline, Serine, Threonine) *de novo* from glucose and inorganic substrates. Also, *T. gondii* can produce 8 AAs, Tyrosine, Glycine, Lysine, Valine, Leucine, Isoleucine, Methionine, Phenylalanine, but require specific

precursors. Relevant to our study is the ability of *T. gondii* to synthesize Phe through the shikimate pathway and adequate supply of a specific precursor, phenylpyruvate, from infected host cell [6]. Despite a capacity for *de novo* synthesis *T. gondii* still scavenges Phe and almost all the amino acids, which are essential for the host, with the exception of threonine. Genomic data indicate that *T. gondii* has the potential to synthesize threonine using homoserine as precursor.⁸ However, a recent study showed that the whole pathway from aspartate to threonine might not be functional in certain life cycle stages and hence *T. gondii* could be a conditional threonine auxotroph [9].

In this study we leveraged the capabilities of a combined stable-isotope labelled Phe and Raman micro-spectroscopy approach to investigate the transport of Phe from host cell to live *T. gondii*, both in time- and spatial-domains. Specifically, we examined the relationship among the processes of Phe transport, possible pool build up, and the incorporation of Phe into newly synthesized protein. It is tempting to speculate that the rate of Phe transport would be correlated to functions, which change during the parasite growth, such as increasing surface area or volume of the growing parasite. However, the time taken for transport of L-Phe(D8) to *T. gondii* was remarkably short. *T. gondii* tachyzoites acquired L-Phe(D8) from host cells at a very rapid rate (~50% at 2-3h PI) and L-Phe became completely substituted with L-Phe(D8) at ~7-9 h PI, which even precedes parasite division.

Upon entering the host cells, the invading *T. gondii* are enclosed within a PV. This intracellular lifestyle, by sequestering within the PV, although it offers

advantages to *T. gondii* by preventing exposure to host cell antimicrobial and immune defences it also separates the metabolic and biosynthetic machinery of the parasite from the nutrient-rich host cytoplasm. Uptake of L-Phe(D8) might consist of active transport through transporters in the parasite membrane into a free AA internal pool. L-Phe(D8) may then be incorporated into new protein. However, the uptaken L-Phe(D8) was not found to be metabolized/cleaved into Tyr by the parasite. This conclusion was supported by the lack of spectral changes related to the Fermi resonance that is expected to be removed upon substitution of H with D in Tyr. [28]. Tyr levels could be below the limit of quantification for the analytical approach used in the present study. These results suggest that L-Phe(D8) uptaken by the *T. gondii* might be incorporated into proteins without conversion to Tyr, similar to previous reports that only low level of turnover of Phe into Tyr was observed when mammalian cells are grown in Tyr-containing culture media [29]. Unexpectedly, the extracellular *T. gondii* tachyzoites which require high energy to empower their gliding motility and invasion into new host cells were found incapable of utilizing L-Phe(D8) from culture medium even after 24 h.

These findings have some biological relevance. The rapid accumulation of L-Phe(D8) within *T. gondii* supports the role of *T. gondii* in behavioral changes in mice or psychiatric behaviors in infected humans [30-33]. The mechanism(s) by which *T. gondii* causes these changes been suggested to be due to the effect of the parasite on neurotransmitter equilibrium, especially dopamine *in vivo* and *in vitro* [32,34]. This hypothesis has been supported by the discovery of two unique genes, *AAAH1* and *AAAH2*, in *T. gondii* genome encoding for two

isoforms of aromatic amino acid hydroxylases *AAAH* (tyrosine and phenylalanine hydroxylases), which catalyze phenylalanine to tyrosine and tyrosine to 3,4 dihydroxyphenylalanine (L-Dopa) (the precursor to dopamine), which may alter dopamine pathways in the brain of infected host, leading to alteration in the behavior of *T. gondii*-infected host [12]. *AAAH1* and *AAAH2* differ in their temporal expression. *AAAH1* was reported to be constitutively expressed across the parasite life cycle, while the expression of *AAH2* was reported to be expressed during the dormant bradyzoite stage [12]. This pattern has led to the assumption that *AAH2* is the prime candidate effector of the parasite's manipulation of host dopamine. Interestingly, a recent study by Wang et al. reported an alternative role of the parasite-encoded *AAAH* enzymes, which was not found to alter DA in the host brain [35]. This, to some extent, agrees with our findings that the uptaken L-Phe(D8) was not metabolized into Tyr by the parasite. This reduction in Tyr during *T. gondii* infection might have a biological reasoning. Patients suffering from chronic immune activation and inflammatory conditions, which are prominent features in toxoplasmosis, were found to have elevated serum levels of Phe together with elevated phenylalanine to tyrosine ratios (Phe/Tyr), which were associated with neuropsychiatric symptoms, such as depression and mood changes [36]. The link reported between immune and inflammatory activation and the disturbed Phe metabolism is most likely a consequence of a reduced conversion of Phe to Tyr by the aromatic amino acid phenylalanine 4-hydroxylase (PAH). Given the oxidative stress associated with these conditions the one would expect that metabolites, such as 5,6,7,8-tetrahydrobiopterin (BH₄), which acts as an antioxidant as well as a cofactor for PAH in mammals are rapidly destroyed [37]. As a consequence,

the BH₄-dependent enzyme PAH loses its activity, leading to increase in Phe concentration and of Phe/Tyr ratio.

The rapid uptake of L-Phe(D8) by *T. gondii* indicate the significant need for this AA by *T. gondii* even at an early stage of the intracellular infection cycle. Also, the two predicted AAAH genes in *T. gondii* reflect the parasite's ability to internalize and digest Phe from the host [12]. In the present study the amount of L-Phe(D8) uptaken from the host cells and incorporated into protein exceeds the expected amounts of new protein formed based on the expected increase in size of the growing *T. gondii* as indicated by the bright field images. It is reasonable to hypothesize that L-Phe(D8) may be transported into *T. gondii* and enter the amino acid pool of the host cell, which is then exchanged with the original L-Phe residues in its native form and without its preliminary degradation similar to the exchange reaction in bacteria [29]. Another possible mechanism that may facilitate the rapid uptake of L-Phe(D8) is the release of proteins by *T. gondii* into the host cells during the early stage of infection. A range of proteins delivered from rhoptries and dense granules, which are secretory organelles unique to the phylum Apicomplexa, are released by *T. gondii* in the initial stages of infection to subvert host cell function [38]. These proteins are secreted into the PV immediately after invasion, and can be detected in the host cell cytosol within 10 min of invasion [39]. Even though the concentration of L-Phe was expected to be very low given the large volume of the mammalian cells spectral maps corresponding to the 1004 cm⁻¹ Raman band assigned to L-Phe molecules was detected inside the host cytoplasm at 2-5 h PI.

T. gondii and other protozoans can only synthesize a few AAs because they have lost key enzymes needed to make some AAs *de novo*. Hence, they rely on host to supply them with AAs, the transport of which is mediated by several plasma membrane carriers (TransportDB). *T. gondii* amino acid hydroxylases enzymes have an N-terminal signal peptide and localize to the parasite plasma membrane and parasitophorous vacuole. Hence, it is very likely that the import of Phe via the plasma membrane and the subsequent entry within the intravacuolar parasites occurs via transporters. Even though AA import is essential for protozoans only a few AA transporters have been functionally characterized, including the AAP (amino acid/auxin permease) transporter family whose number vary significantly among protozoan species, and may transport one or multiple different AAs, reflecting fundamental differences among protozoan species in AA requirements or salvage mechanisms [40]. However, we calculated the transfer rate of L-Phe(D8) molecules from host to the *T. gondii* to be $0.5-1.6 \times 10^4$ molecules/s. Because an average transporter can have a transport rate of $\sim 10^3-10^4$ molecules/s, our results agree with the prediction that four AAP transporters are involved in the AA transfer in *T. gondii* B7 strain (TransportDB). On the other hand, two transport systems for Phe have been identified in the protozoan *Trypanosoma brucei brucei*, characterized by a high and a very high affinity [41]. When we expressed the Vmax values in terms of molecules/s/single cell, we estimated transport rates of 9.4×10^4 and 56.4×10^4 of Phe molecules/s for the high and the very high transport system, respectively. These transport rates are 10 to 50-fold higher than the rates for *T. gondii* measured in our study. Overall, these data support the idea that differences exist in the trafficking of Phe between different protozoans.

5.6 Conclusions

Common imaging techniques often do not allow distinguishing between newly imported AAs and those already existing within the cell, or visualizing individual AA. To overcome these challenges, we used a Raman microspectroscopy-based imaging approach to directly monitor the uptake of individual deuterium-labeled Phe by individual *T. gondii*. Using this approach, we show for the first time that it is possible to follow in real time the uptake dynamics and intracellular distribution of L-Phe(D8) within individual live *T. gondii*-infected cells. *T. gondii* tachyzoites acquired L-Phe(D8) from pre-labelled host cells at a very rapid rate (~50% at 2-3h PI) and L-Phe became completely substituted with L-Phe(D8) at ~7-9 h PI. Based on calibration model, it was also possible to estimate the transfer rate of L-Phe(D8) molecules from host to the *T. gondii* to $0.5-1.6 \times 10^4$ molecules/s. Maintenance of some metabolite pools in *T. gondii* may be a result of both uptake and *de novo* production. Phe, whether supplied by the host cell or produced endogenously by the parasite, is among the nutrients required for the growth of both the host cell and the parasite. Therefore, accurate consideration of metabolism in the host and pathogen together is central for a better understanding of the metabolic needs and capabilities of *T. gondii*. Although the import of Phe by the host cell plasma membrane has been documented the transporter(s) responsible of the import of Phe from host cell to the parasite growing within the PV has not yet been precisely identified. Future analyses should address whether depletion or disruption of Phe salvage pathway in *T. gondii* may hold a therapeutic perspective. Our approach can facilitate the study other intracellular microbial

systems in which metabolic exchanges between the host cell and the intracellular pathogen are prominent.

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Chapter 6

Tracking amino acid's uptake into the protozoan *Acanthamoeba castellanii* by stable-isotope labelling and Raman spectral imaging

The capacity of pathogens to acquire nutrients from their host cells is one of the most fundamental aspects of infection biology. Hence, measuring the patterns of nutrients' uptake by pathogens is essential for understanding the interactions of pathogens with eukaryotic host cells. In this part, we optimized a technique that allows fast and non-destructive measurement of the amino acid Phenylalanine (Phe) acquired by the trophozoite stage of the protozoan *Acanthamoeba castellanii* (*A. castellanii*) as they engage with individual human retinal pigment epithelial cells (ARPE-19). ARPE-19 host cells were pre-saturated with Deuterated Phe (L-Phe(D8)) to replace the native substrate Phe (L-Phe). The uptake of L-Phe(D8) by *A. castellanii* trophozoites was measured by Raman microspectroscopy. This approach allowed us to characterize the uptake patterns of this essential amino acid into *A. castellanii* trophozoites at a single cell level. At 24 hours post infection (PI) *A. castellanii* trophozoites are capable of salvaging L-Phe(D8) from host cells. The uptake pattern was time-dependent during the first 24 hours of infection and complete substitution with L-Phe(D8) in all parasites was detected at 48 hours PI. On the other hand, isolated *A.*

castellanii trachyzoites (grown without host cells) did not show significant uptake for L-Phe(D8) from the media; only achieved an uptake ratio of 16-18% of L-Phe(D8) from the culture medium after 24 hours. These findings demonstrate the potential of combining Raman microspectroscopy and stable isotope labelling approaches to elucidate the role of metabolism in mediating *A. castellanii* interaction with host cells.

6.1 Introduction

A. castellanii exists in the vegetative, infective and reproducible trophozoite stage under favourable conditions [1,2]. Under physiological stress i.e. extreme temperature, osmolarity, pH and lack of nutrients or desiccation, trophozoite transforms into a metabolically inactive cyst [3] and encloses itself within a thick wall [2] and becomes resistant to biocides, chlorination and antibiotics [4]. Cysts return to their trophozoite with improved conditions in a process known as excystment [5]. Diseases caused by *A. castellanii* are not well understood despite their debilitating and life-threatening consequences due to various experimental and conceptual challenges. Some pathogenic mechanisms have been partially characterized, however in many cases better description is yet to be achieved. Raman microspectroscopy has also been used with SILAC to differentiate the cellular components produced through distinct metabolic pathways [6,7,8,9], lipid uptake and metabolism inside human macrophages on single cell level [10], proteome localization in single Fission Yeast Cells [11].

The aim of this study was to employ RMS coupled with isotope labelling to characterize the uptake of the Deuterated Phenylalanine (L-Phe(D8)) by *A.*

castellanii trophozoites from ARPE-19 host cells. Results showed that trophozoites acquired L-Phe(D8) from host cells soon after infection, suggesting that such an arrangement is metabolically beneficial for this organism.

6.2 MATERIALS AND METHODS

6.2.1 Cell line and culture conditions

The human retinal pigment epithelial cells (ARPE-19) were used at passage 21. Cell Culture is explained in Chapter 4, section 4.1.1. As an immune-privileged and easily accessible organ, the eye constitutes a favourable target for *Acanthamoeba castellanii*. ARPE-19 cell line was selected as a model to investigate the molecular changes that accompany *A. castellanii* infection to the ocular surface.

6.2.2 The parasite strain

A clinical isolate of *Acanthamoeba castellanii* belonging to the T4 genotype, originally obtained from the American Type Culture Collection (ATCC #50492), was used in the study. Amoebae were grown in T-175cm² tissue culture flasks (Sarstedt, UK) in autoclaved axenic peptone-yeast-glucose (PYG) medium comprising 0.75% w/v proteose peptone (Oxoid™, Thermo-Fisher Scientific, UK), 0.75% w/v yeast extract, and 1.5% w/v glucose in distilled water. Flasks were maintained in a humidified standard air incubator at 30°C. Every 3-5 days the growth medium was removed from culture flasks and replaced with fresh PYG. Under these conditions more than 90% of *A. castellanii* remained bound to the flask as trophozoites.

6.2.3 The doubling time (DT) of cells ARPE-29

The doubling time (DT) of ARPE-19 cells grown *in vitro* was determined by an experiment performed for a period of 6 days. Cells were grown in a six-well plates and counted every 24 hours via cell counting using a haemocytometer and disposable plate. The cells density kept on increasing from the first day to fifth day. On Sixth day, it was less dense than fifth day, as the cells occupied all the space in the well plates and they start dying.

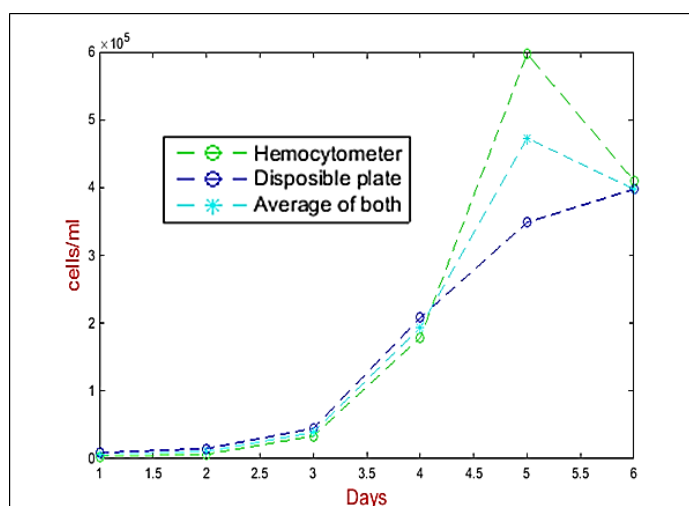


Figure 6.1: The doubling time (DT) of ARPE-19 cells grown *in vitro* was determined every 24 hours for a period of 6 days via cell counting using a haemocytometer and Disposable cell counting plate. DT is found to be average of 24 hours from the data.

From day 1, 2 and 3, the doubling time was found to be approx. 24 hours, from day 4 and 5 it was 24 hours. The cell density should be 100cells/each case counted to determine the doubling time of the cell line. The data from the day 1, 2, 3 have higher level of uncertainty as compared to the day 4 and day 5.

Therefore, it was taken as 24 hours from the average values. The cell counting data is shown in Figure 6.1.

6.2.4 Stable isotope labelling by amino acids (SILAC)

The aim of this experiment was to determine the molecular exchange between host ARPE-19 cells and *A. castellanii* trophozoites. The ARPE-19 cells are cultured in a specially-formulated medium containing all amino acids (AA) in unlabelled form, but Phenylalanine (Phe), which was added with the desired isotope label. To minimize the background signal of the L-Phe ARPE-19 cells were synchronized overnight in serum-deficient, Phe-free customised DMEM culture medium. The cells were subsequently incubated with SILAC DMEM medium supplemented with L-Phe(D8). After complete substitution of L-Phe by L-Phe(D8) in ARPE-19 cells, the cells are challenged with *A. castellanii* trophozoites as described below.

6.2.5 Infection of host cells with *A. castellanii*

ARPE-19 cells (10^4 cells/ml) saturated with L-Phe(D8) were seeded in titanium cell-chambers, which are fitted in 6-well plastic cell culture plates, with a volume of 2 ml DMEM/chamber. The sample holders (titanium cell chambers) were purpose-built to enable acquisition of Raman spectra of the cells and incorporated MgF2 coverslips (0.17 mm thick) at the bottom. Before introducing the parasites to ARPE-19 cells the culture medium was replaced with L-Phe-free media to avoid any false labelling of the parasite. Parasites are grown in PYG medium, but they were transferred to L-Phe-free DMEM media during host cell infection. The only source of L-Phe(D8) to the parasites comes from the host

cells and L-Phe for host cells are the parasites. Infection was initiated by adding *A. castellanii* trophozoites at a multiplicity of infection (MOI) of 1 cell to 1 parasite (1C:1P). The procedure of infection protocol is explained diagrammatically in Figure 6.2. Key challenge to the imaging of live *A. castellanii* trophozoites is the speed at which trophozoites of this organism move. During live Raman spectral mapping, *A. castellanii* trophozoites were found to move beyond the designated area of imaging before the completion of raster scan measurement at 0.5 seconds acquisition time. Thus, we had to perform the measurements on trophozoites fixed with 4% paraformaldehyde before starting the Raman spectral imaging.

6.2.6 L-Ph(D8) uptake by isolated *A. castellanii* trophozoites

We tested the ability of isolated trophozoites to acquire L-Phe(D8) from the medium by harvesting 2×10^7 *A. castellanii* trophozoites from cultured flasks, followed by washing in 10ml of sterile Dulbecco's phosphate-buffered saline (DPBS) three times to remove any traces of PYG medium. The trophozoite pellet was suspended in 2 ml of L-Ph(D8)-supplemented medium for 24 hours and were subjected to Raman imaging.

6.2.7 Raman imaging

The laser is focused through a MgF2 coverslip at the bottom of specially designed sample holders. A 785 nm ~ 170 mW laser (before objective) (Ti:sapphire laser, spectra-physics) was used for excitation of Raman spectra.

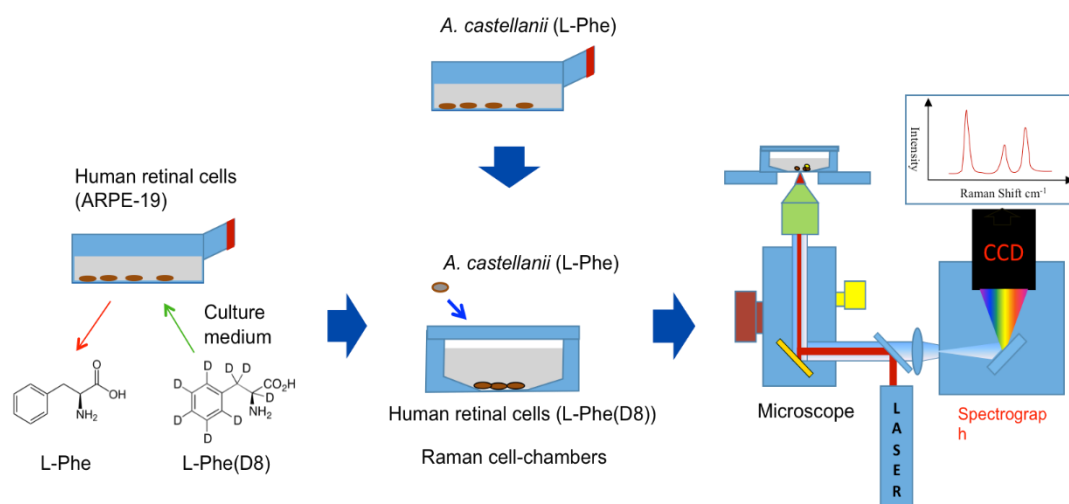


Figure 6.2: Human retinal cells (ARPE-19) were cultured in SILAC DMEM media to replace L-Phe with L-Phe(D8). After complete saturation with L-Phe(D8), ARPE-19 cells were challenged with *A. castellanii* (grown in L-Phe medium). After creating infection, sample holders are placed under Raman microspectrometer for Raman imaging.

6.2.8 Fluorescence staining

Acridine orange (AO) staining method was explained in Chapter 3, section 3.4.2. AO fluorescence imaging of *A. castellanii*-infecting cells was performed, on the same cell, after completion of the Raman spectral imaging. Imaging was performed by using wide-field fluorescent microscope integrated on the confocal Raman micro-spectrometer. The retrospective positioning and identification of the cells was based on two thin marks engraved on the cell chambers (retro-positioning accuracy was 5 μm).

6.3 Image analysis and data processing

Data pre-processing involved cosmic ray removal and background subtraction. Noise in the resulting data matrix was reduced by using singular value decomposition. The spectral images presented in Figures 6.3, 6.4, 6.5 were

obtained by calculating the height of the specified Raman bands after subtraction of a local linear baseline.

6.4 RESULTS

6.4.1 Uptake of L-Phe(D8) by free *A. castellanii* from SILAC media

In order to investigate the intake of L-Phe(D8) from the culture medium, *A. castellanii* trophozoites were grown in normal medium conditions containing L-Phe and were subsequently incubated in media supplemented with L-Phe(D8). After 24 hours of incubation of *A. castellanii* in the L-Phe(D8)-supplemented medium, Raman spectral imaging was performed. The Raman maps are plotted to represent lipids (1449cm^{-1}), L-Phe (1004 cm^{-1}), and L-Phe(D8) (960 cm^{-1}) inside *A. castellanii* as shown in Figure 6.3. The uptake ratio of L-Phe(D8) was calculated by considering peaks heights of L-Phe (P_{1004}) and L-Phe(D8)(P_{960}) by using the formula $(P_{960}/(P_{1004}+P_{960}))*100$ and these results show that the uptake of L-Phe(D8) from media in 24 hours was 16-18% as compared with the total amount of Phe in the parasites.

The Raman spectra of *A. castellanii* (Fig. 6.3c) have bands corresponding to lipids, proteins and nucleic acids. The most intense bands are assigned to lipids: C=C stretching 1660 cm^{-1} , CH_2 deformation 1449 cm^{-1} , CH_2 twisting 1303 cm^{-1} , =C–H deformations 1260 cm^{-1} , symmetric stretching of $\text{N}(\text{CH}_3)_3$ of choline group 719 cm^{-1} , nucleic acids (O–P–O) 788 cm^{-1} , PO_2 1080 cm^{-1} , and the ring vibrations of protein phenylalanine 1004 cm^{-1} .

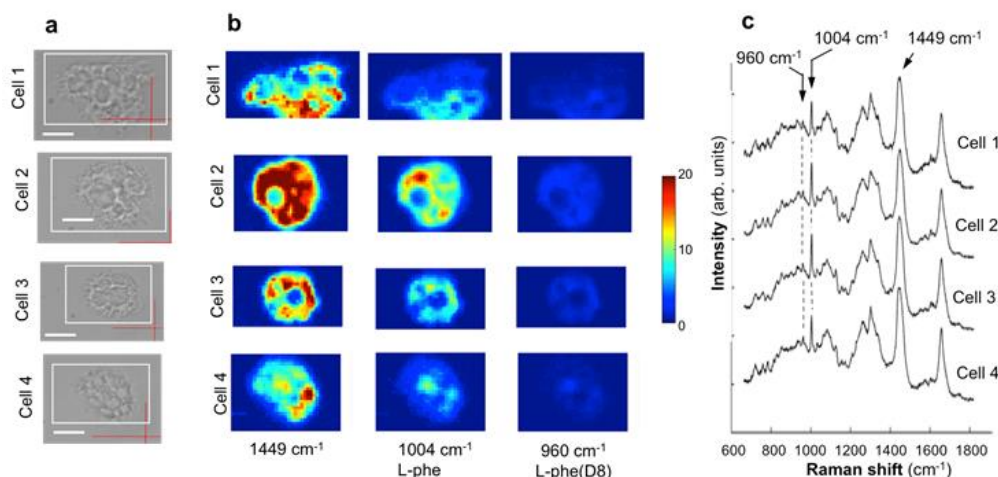


Figure 6.3: Raman spectral imaging of *A. castellanii* at 24 hours of incubation in L-Phe(D8)-supplemented media. (a) Bright field, (b) Maps corresponding to Raman spectral peaks 1449 cm^{-1} , 1004 cm^{-1} , 960 cm^{-1} , (c) Average Raman spectra of *A. castellanii* trophozoites. Scale bars: 10 μm .

6.4.2 Uptake of L-Phe(D8) by *A. castellanii* from ARPE-19 cells

ARPE-19 cells grown in experimental chambers were inoculated with *A. castellanii* trophozoites. After 24 hours of PI, the interacting parasites and cells are measured by Raman raster scanning. Raman spectra and spectral maps corresponding to the L-Phe(D8) 960 cm^{-1} and L-Phe 1004 cm^{-1} of ARPE-19 cells and *A. castellanii* parasites are shown in Figure 6.4 to show the interchange of L-Phe(D8) between ARPE-19 cells and parasites. Intense Raman bands corresponding to L-Phe(D8) at 960 cm^{-1} can be observed at 24 hours PI in the spectra of *A. castellanii* trophozoites, with a decrease in the intensity of the L-Phe 1004 cm^{-1} for the samples 1 and 2 as shown in Figure 6.4. *A. castellanii* in sample 3 Figure 6.4 does not show any sharp peak of 960 cm^{-1} and a Raman band at 1004 cm^{-1} can be detected in the APRE-19 cell that shows the release of proteins by *A. castellanii* into the host. It is also interesting that one of *A.*

castellanii trophozoite in sample 4 in Figure 6.4 was completely saturated with L-Phe(D8) at 24 hours PI.

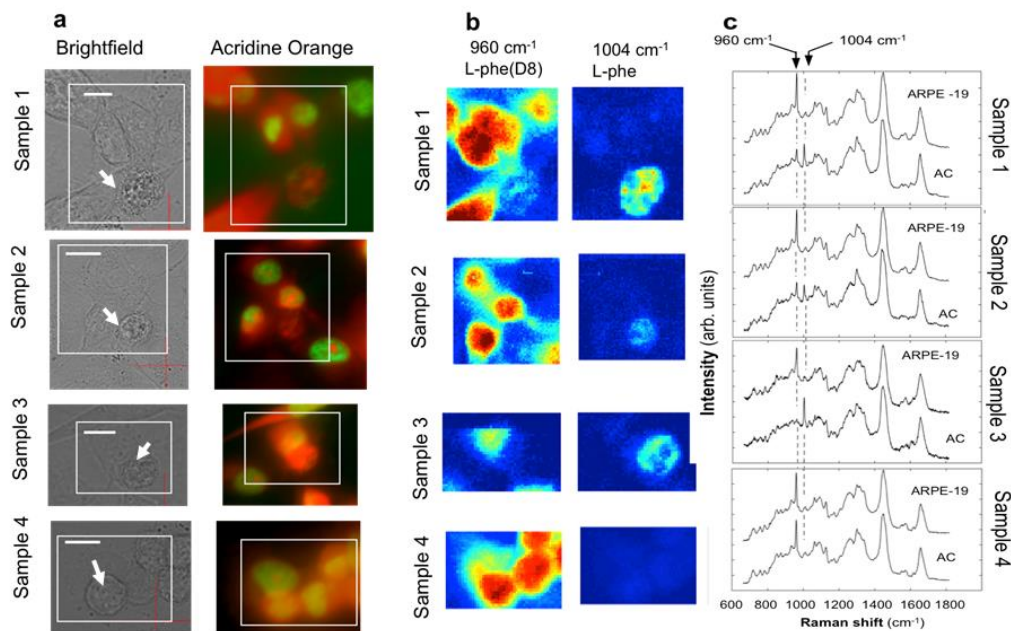


Figure 6.4: Raman spectral imaging of ARPE-19 cells infected with *A. castellanii* at 24 hours PI (a) Bright field images and fluorescence images (recorded immediately after Raman spectroscopy measurements), white arrows are drawn to show the position of *A. castellanii* inside host cells. (b) Maps corresponding to Raman spectral peaks 1004 cm⁻¹, 960 cm⁻¹. (c) Average Raman spectra from *A. castellanii* trophozoites and ARPE-19 cells. Scale bars: 10 μm.

ARPE-19 cells infected with *A. castellanii* are measured after 48 hours PI and the spectral maps and Raman spectra are shown in Figure 6.5. All *A. castellanii* trophozoites at 48 hours of infection are found completely substituted with L-Phe(D8). Raman band for the L-Phe 1004cm⁻¹ band is no longer detected in the Raman spectra of *A. castellanii* trophozoites, suggesting that *A. castellanii* acquired L-Phe(D8) from the host cells.

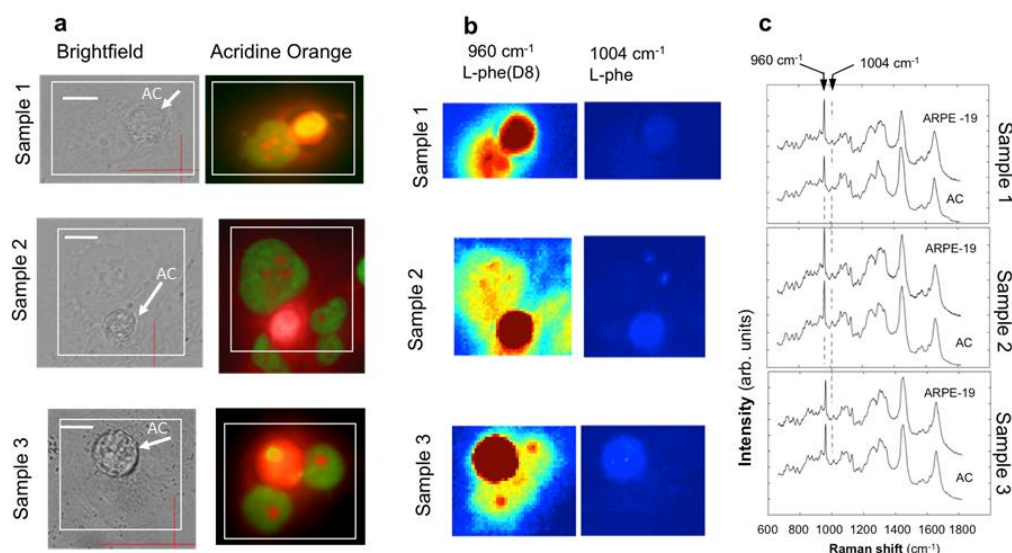


Figure 6.5: Raman spectral imaging of ARPE-19 cells infected with *A. castellanii* at 48 hours PI), white arrows are drawn to show the position of *A. castellanii* inside host cells. (a) Bright field images and fluorescence images (recorded immediately after Raman spectroscopy measurements), white arrows show the position of *A. castellanii* inside host cells (b) Maps corresponding to Raman spectral peaks 1004cm⁻¹ and 960cm⁻¹. (c) Average Raman spectra from *A. castellanii* trophozoites and ARPE-19 cells. Scale bars: 10 μ m.

6.4 DISCUSSION

The aim of this study was to address how *A. castellanii* trophozoites breach the human retinal pigment epithelial cells (ARPE-19) cells, which represent the main constituent of the blood-retinal barrier (BRB) from a metabolic perspective. We investigated mechanisms of pathogenesis in this infection model of human retina and focussed on a new way by which amoebae interact with these host cells. To facilitate this we employed, for the first time, stable isotope labelling of the amino acid Phe and Raman spectroscopy techniques to interrogate amoeba's ability to acquire Phe from the host cells. This approach allowed us to investigate the relative importance of metabolic exchange between *A. castellanii* and its host cells to BRB *in vitro*.

These results show that extracellular *A. castellanii* trophozoites are not capable of complete uptake of L-Phe(D8) from culture medium. The peak related to isotope labelled L-Phe(D8) 960cm^{-1} is very small even at 24 hours of incubation in SILAC DMEM media. On the other hand, when *A. castellanii* trophozoites are introduced to ARPE-19 cells, they showed fast uptake of L-Phe(D8), that was solely sourced from the host cells, as the medium used does not contain L-Phe. All of the *A. castellanii* trophozoites measured at 24 hours of PI showed uptake ratios between 40-60% of L-Phe (D8) from host cells. Only one interacting parasite-host system in sample 3 Figure 6.4 was found, in which we detected L-Phe 1004cm^{-1} peak in the ARPE-19 cell, but the parasite did not show any peak for L-Phe(D8). This suggests that the parasite might be releasing some sort of protein into host cell as an early step of trophozoite-ARPE-19 cell interaction [12,13]. In Figure 6.4, sample 4, *A. castellanii* trophozoites was completely saturated with L-Phe(D8) at 24 hours PI. These results demonstrate that uptake ratio of Phe depends on the physical contact between the host and the parasite. The importance of binding in the initiation of *A. castellanii* infection has been demonstrated in a variety of model systems, showing that when adhesion is blocked (primarily by exogenous mannose) the parasite's ability to kill corneal or endothelial host cells is reduced [13,14]. Perhaps, parasites that are able to engage with host cells at the start of the experiment are the ones detected with complete substitution of L-Phe(D8) at 24 hours PI. At 48 PI, all the parasites were found completely saturated with L-Phe(D8).

Taken together, using Raman cell imaging coupled with SILAC, we showed, for the first time, that *A. castellanii* tachyzoites can acquire L-Phe(D8) from pre-

labelled host ARPE-19 cells at a rate (~40-60% at 24 hours PI) in most of the parasites and L-Phe became completely substituted with L-Phe(D8) at 48 hours PI. On the other hand, *A. castellanii* trophozoites did not show more than 18% uptake ratio of L-Phe(D8) even after 24 hours of incubation in SILAC DMEM medium, suggesting that although *A. castellanii* trophozoites can exist in a free-living status but their interaction with host cells provide metabolic and nutritional needs to the parasite.

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Chapter 7

Conclusions

7.1 Summary of work

The work presented in this thesis has been conducted to meet the need for better understanding, at a molecular level, of the host pathogen interaction. The experimental work presented here demonstrates strongly the potential of Raman spectroscopy to detect molecular changes and *in-situ* monitoring of molecular dynamics during host–pathogen interaction. The thesis is divided in to six chapters, with the first chapter based on the introductory information, second chapter to the brief review about the techniques used for analysis of cells and Raman spectroscopy in the pathogens study. The third chapter briefly describe the Raman micro-spectrometer, evaluation of the instrument, in terms of spectral and spatial resolution, calibration and performance of the integrated florescent Imaging system.

Chapter 4 describes the first study made on human retinal pigment epithelial cells (ARPE-19) infected with *T. gondii* by using Raman micro-spectroscopy. The study was done on fixed and live cells infected with the *T. gondii* at different post infection time points. The results indicated that significant increase in the concentrations of proteins and lipids was found with the increase in the time of

infection. There was also an increase in nucleic acids as early as 6 hours post infection, with the development of *T. gondii* tachyzoites inside cells.

As the study presented in Chapter 4 does not pinpoint the specificity of the molecules responsible for these changes, further experiments were undertaken to specifically monitor the changes related to amino acids in the host cells and divided parasites.

Chapter 5 continued the work on the infection caused by the *T. gondii* tachyzoites in human retinal pigment epithelial cells (ARPE-19) focusing on the exchange of the amino acid phenylalanine (Phe). In this study, the combined approach of SILAC and Raman microspectroscopy was used to distinguish between the newly imported amino acids and those already existing within the cells and parasites. Host cells used are isotope-labelled with L-Phe(D8) and parasites are grown in L-phe(H8) media before creating infection. In this study, it is showed for the first time that it is possible to follow in real time the uptake dynamics and intracellular distribution of L-Phe(D8) within individual live *T. gondii*-infected cells. *T. gondii* tachyzoites acquired L-Phe(D8) from host cells at a very rapid rate (~50% at 2-3h hours of post infection) and became completely substituted L-phe(H8) with L-Phe(D8) at ~7-9 hours of post infection. The transfer rate of L-Phe(D8) molecules from host to the *T. gondii* was estimated to be $0.5-1.6 \times 10^4$ molecules/sec.

In chapter 6, the mechanism of interaction of extracellular trophozoites of *A. castellanii* was studied with the human retinal pigment epithelial cells (ARPE-19). In this study, pre-labelled host cells (ARPE-19) with L-phe(D8) were treated

with *A. castellanii* trophozoites containing L-Phe(H8). The results at 24 hours post infection indicate that the uptake ratio of 40-60% of L-Phe (D8) from host cells to the *A. castellanii* trophozoites in most parasites and complete substitution of L-Phe(H8) with L-Phe(D8) in *A. castellanii* trophozoites at 48 hours of infection suggesting that such an arrangement is metabolically beneficial for this organism.

The *A. castellanii* trophozoites were also grown in SILAC media containing L-Phe(D8) without introducing to the cells to see the uptake of L-Phe(D8). These parasites showed uptake ratio of 18% of L-Phe(D8) even after 24hours in the media. These results shows that physical engaging of extracellular parasites *A. castellanii* trophozoites and host cells is important for the metabolic exchange between them.

The work presented in these experimental chapters 5 and 6 describes the first study made by using the combined Stable isotope labelling by amino acids in cell culture (SILAC) and Raman micro-spectroscopy to study the amino acids exchange between host-intracellular *T. gondii* and host-extracellular *A. castellanii* parasites.

7.2 Future research direction

SILAC combined with Raman micro-spectroscopy have been used to selectively monitor the incorporation of deuterium-labelled Phe into proteins in individual live tachyzoites. Future studies are still required to identify the transporter(s) responsible for the import of Phenylalanine from host cell to the parasite growing

within the parasitophorous vacuole. Also studies can be performed to see whether depletion or disruption of Phe can affect the behavioural changes in *T. gondii*-infected host.

This work could have significant implications in unravelling unknown metabolic processes mediating host-pathogen interactions. This approach can also be used to study other intracellular microbial systems during host-pathogens interactions. The combined SILAC-Raman approach is a versatile method that can facilitate new applications in cell biology.