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

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ABSTRACT

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 study, 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.

Key words: *Acanthamoeba castellanii*; host-pathogen interaction; Raman Spectroscopy

1. INTRODUCTION

Acanthamoeba species, discovered in 1930, are the most prevalent free-living protozoa and inhabit a variety of environments including air, soil and water[1], swimming pools[2], eye wash stations[3], and noses and throats of asymptomatic individuals[4]. *Acanthamoeba* exists in two different life forms: trophozoite and cyst. Under favourable conditions i.e. abundant food supply, neutral pH, appropriate temperature and osmolarity, *Acanthamoeba* exists in the vegetative, infective and reproducible trophozoite stage. This form of the life cycle has a diameter of 10-25μm[5] and exhibits irregular shape with locomotor pseudopods and surface acanthopodia[6,7]. Under physiological stress i.e. extreme temperature, osmolarity, pH and lack of nutrients or desiccation, trophozoite transforms into a metabolically inactive cyst[8] and encloses itself within a thick wall[7] and becomes resistant to biocides, chlorination and antibiotics[9]. Cysts return to their trophozoite form with improved conditions in a process known as excystment[10].

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 particular the metabolic alteration in both amoeba and host cell is an area, which requires further investigation, for example *A. castellanii* access to host nutrients in infected tissues, which is fundamental for the parasite growth, virulence and disease progression. Our understanding of this crucial process is still limited because of various experimental and conceptual challenges. However, the recent application of stable isotope labelling of the aromatic amino acid phenylalanine (Phe) combined

with Raman microspectroscopy (RMS) has enabled the real-time detection of amino-acid's exchange during the interaction of the apicomplexan protozoan parasite *Toxoplasma gondii* with the human retinal pigment epithelial cells (ARPE-19)[11]. This approach allowed real-time tracking of the labeled Phe amino acid non-invasively on a single cell level and with micrometer spatial resolution. RMS has also been used with isotope labelling technology to differentiate the cellular components produced through distinct metabolic pathways[12,13,14,15] , lipid uptake and metabolism inside human macrophages on single cell level[16], and proteome localization in single fission yeast cells[17].

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.

2. MATERIALS AND METHODS

2.1 Cell line and culture conditions

The human retinal pigment epithelial cells (ARPE-19) were used at passage 21. 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. ARPE-19 cells were maintained in DMEM supplemented with 1% penicillin/streptomycin and 5% heat-inactivated fetal bovine serum (FBS). Cells were incubated in 5% CO_2 incubator at 37°C and were passaged twice weekly. Cell viability was determined by trypan blue exclusion assay and cells those have >98% viability were used in the experiments.

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-175cm^2 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.

2.3 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). 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 hemocytometer. 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.

2.4 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 1. 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 0.5seconds acquisition time. Thus, we had to perform the measurements on trophozoites fixed with 4% paraformaldehyde before starting the Raman spectral imaging.

2.5 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.

2.6 Raman imaging

Raman spectra were recorded using a purpose-built confocal Raman micro-spectrometer optimized for cell studies[18] (Figure 1). An inverted microscope (IX71, Olympus, Essex, UK) was used for the setup as it allows cells measurements without the limitation of the objective lens dipping in culture media as opposed to up-right microscopes. 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. To maximize the spatial resolution and collection efficiency for the Raman spectra, a water-immersion 60X objective, NA 1.20 (Olympus) was used for focusing the laser (beam diameter expanded to match the objective pupil, laser spot ~700 nm on sample) on individual cells as well as for collection of Raman scattered photons. The Raman scattered light was collimated and then focused on a 50 mm diameter optical fibre connected to a spectrometer equipped with a 830 lines per mm grating (spectral resolution of 1.5 cm^{-1} in the 600–1800 cm^{-1} region) and cooled deep-depletion back-illuminated CCD detector (Andor Technologies, Belfast, UK). Raman spectral imaging was performed by scanning areas of the cells through the laser focus in a raster pattern (1 micron step size) using a high-precision step-motor stage (Prior, Cambridge, UK) and acquiring Raman spectra at each position (1s per pixel). The spectrometer wavenumber axis was calibrated prior to each experiment using tylenol at an accuracy of 0.5 cm^{-1}.
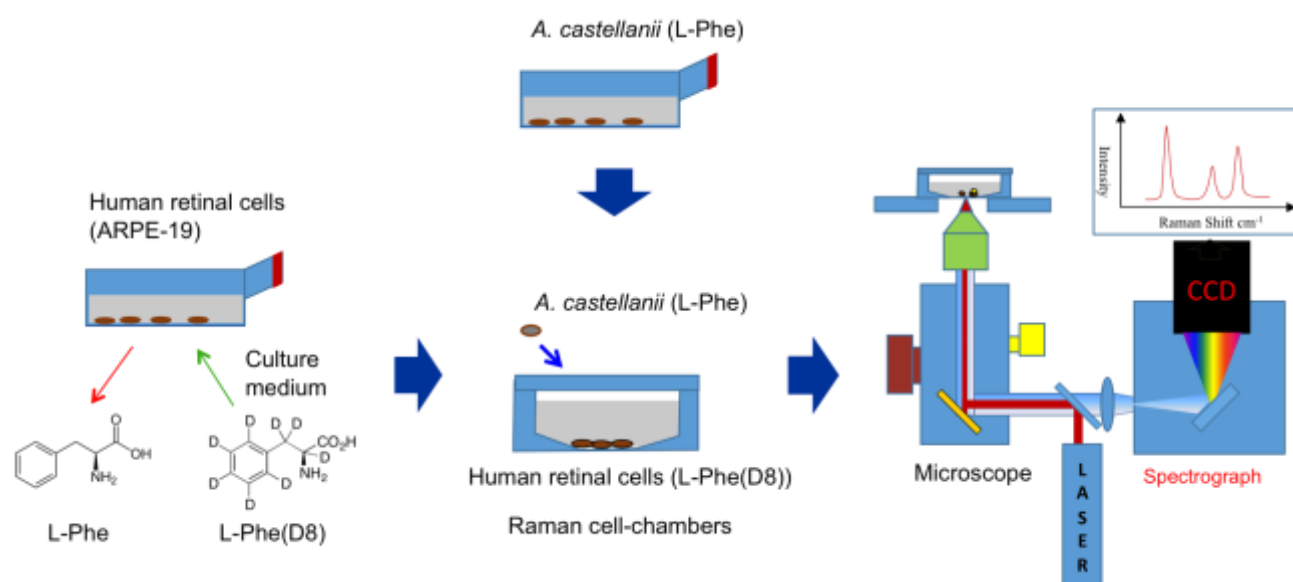
Figure 1: Human retinal cells (ARPE-19) were cultured in SILAC DMEM media to replace L-Phe with L-Phe(D8). After complete saturation with L-Phe(D8), ARPE-19 cells were challenged with *A. castellanii* (grown in L-Phe medium). After creating infection, sample holders are placed under Raman microspectrometer for Raman imaging.

2.6 Fluorescence staining

Acridine orange (AO) staining was used to distinguish the morphological appearance of the *A. castellanii* trophozoites from host cells. AO is a DNA intercalating dye and is used to differentially stain RNA and DNA inside eukaryotic cells. 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. 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).

2.7 Image analysis and data processing

Data pre-processing and analysis was done by using in-house built functions in Matlab (The MathWorks, Natick, MA). Data pre-processing involved cosmic rays removal and background subtraction. Noise in the resulting data matrix was reduced by using singular value decomposition. The spectral images presented in Figures 2, 3, 4 were obtained by calculating the height of the specified Raman bands after subtraction of a local linear baseline.

3   RESULTS

## 3.1 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 2. 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. 2c) have bands corresponding to lipids, proteins and nucleic acids. The most intense bands are assigned to lipids: C=C stretching 1660 cm^{-1}, CH2 deformation 1449 cm^{-1}, CH2 twisting 1303 cm^{-1}, =C–H deformations 1260 cm^{-1}, symmetric stretching of N(CH3)3 of choline group 719 cm^{-1}, nucleic acids (O–P–O) 788 cm^{-1}, PO2 1080 cm^{-1}, and the ring vibrations of protein phenylalanine 1004 cm^{-1}.
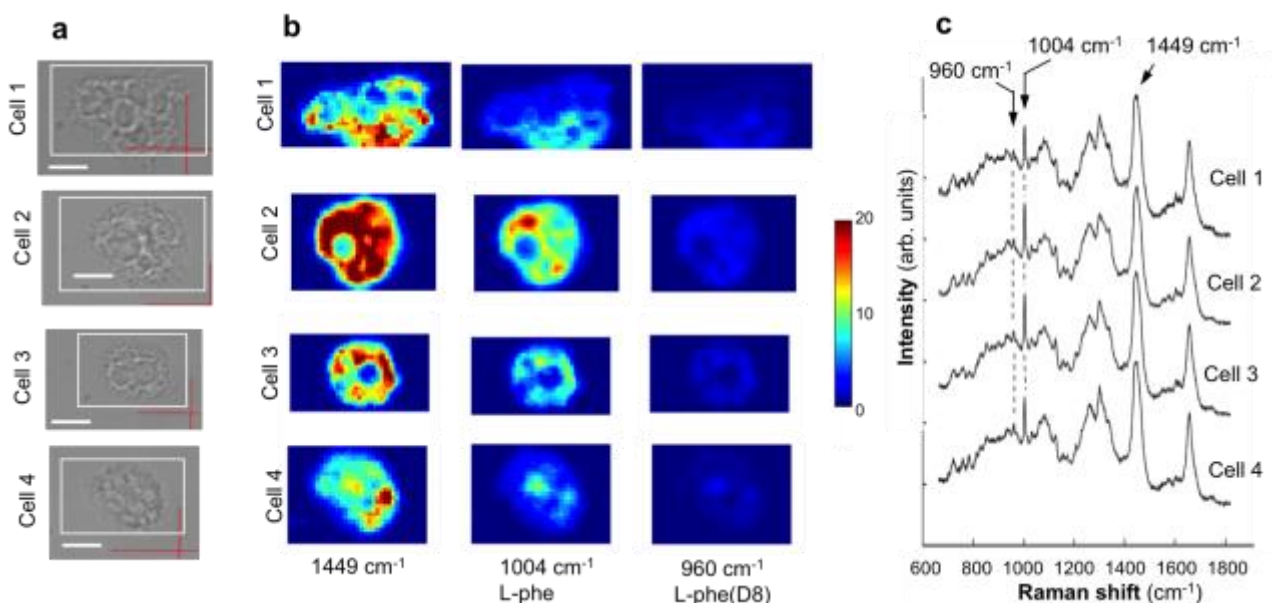
Figure 2. Raman spectral imaging of *A. castellanii* at 24 hours of incubation in L-Phe(D8)-supplemented media. (a) Bright field, (b) Maps corresponding to Raman spectral peaks 1449cm⁻¹, 1004cm⁻¹, 960cm⁻¹, (c) Average Raman spectra of *A. castellanii* trophozoites. Scale bars: 10 μm.

## 3.2 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 3 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 1004cm⁻¹ for the samples 1 and 2 as shown in Figure 3. *A. castellanii* in sample 3 figure 3 does not show any sharp peak of 960cm⁻¹ and a Raman band at 1004cm⁻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 figure3 was completely saturated with L-Phe(D8) at 24 hours PI.
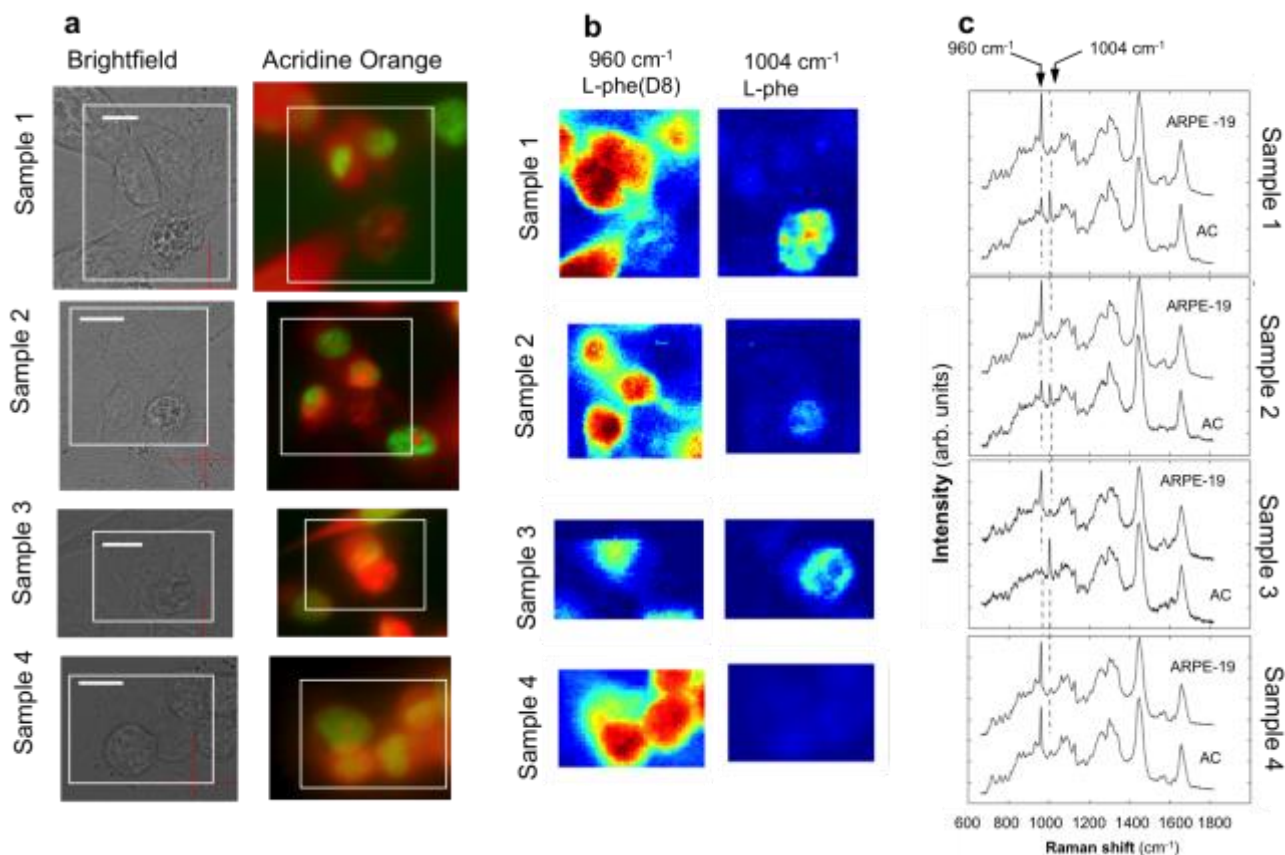
Figure 3. Raman spectral imaging of ARPE-19 cells infected with *A. castellanii* at 24 hours PI (a) Bright field images and fluorescence images (recorded immediately after Raman spectroscopy measurements) (b) Maps corresponding to Raman spectral peaks 1004cm⁻¹, 960cm⁻¹. (c) Average Raman spectra from *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 4. 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.
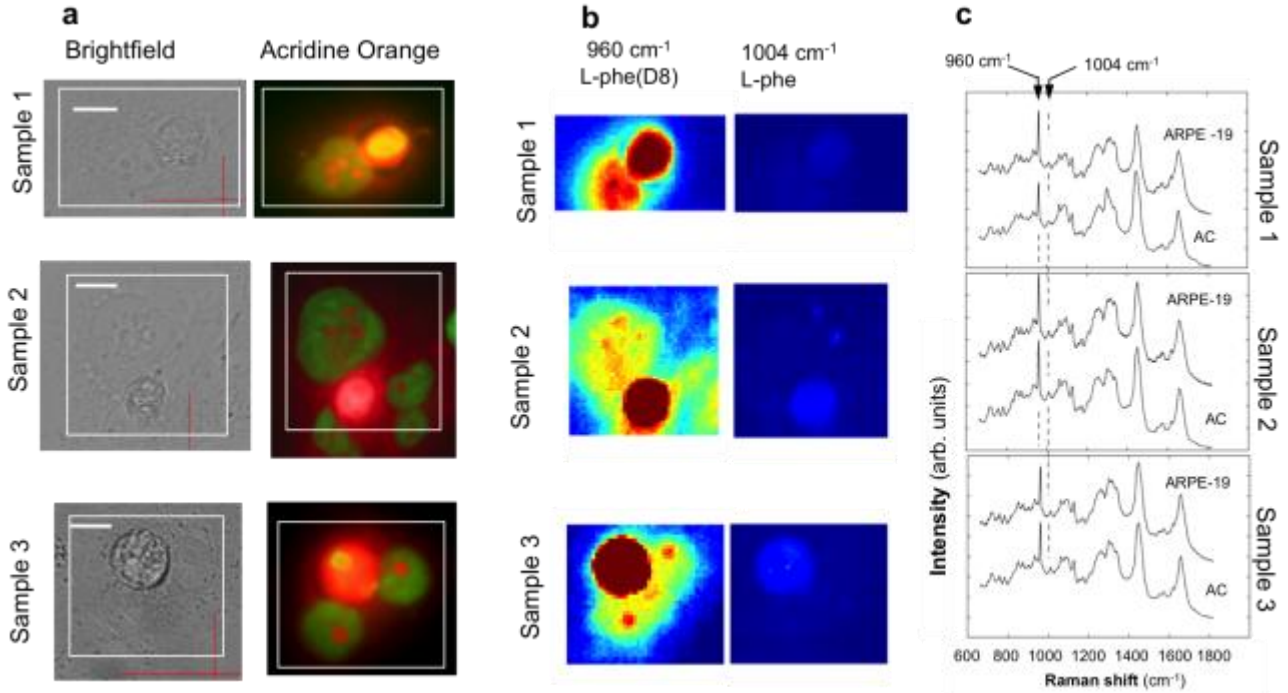
Figure 4. Raman spectral imaging of ARPE-19 cells infected with *A. castellanii* **at 48 hours PI** (a) Bright field images and fluorescence images (recorded immediately after Raman spectroscopy measurements) (b) Maps corresponding to Raman spectral peaks 1004cm⁻¹ and 960cm⁻¹. (c) Average Raman spectra from *A. castellanii* trophozoites and ARPE-19 cells. Scale bars: 10 μm.

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 3 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[19,20]. In Figure 3, 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[20,21]. 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 their interaction with host cells provide metabolic and nutritional to the parasite.

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