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The lag phase and G_1 phase of a single yeast cell monitored by Raman microspectroscopy

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We optically trapped a single yeast cell for up to 3 h and monitored the changes in the Raman spectra during the lag phase of its growth and the G_1 phase of its cell cycle. A non-budding cell (corresponding either to the G_0 or G_1 phase) was chosen for each experiment. During the lag phase, the cell synthesises new proteins and lipids and the observed behaviour of the peaks corresponding to these constituents as well as those of RNA served as a sensitive indicator of the adaptation of the cell to its changed environment. Temporal behaviour of the Raman peaks observed was different in the lag phase as compared to the late lag phase. Two different laser wavelengths were applied to study the effect of long-term optical trapping on the living cells. Yeast cells killed either by boiling or by a chemical protocol were also trapped for a long time in a single beam optical trap to understand the effect of optical trapping on the behaviour of observed Raman peaks. The changes observed in the Raman spectra of a trapped yeast cell in the late G_1 phase or the beginning of S phase corresponded to the growth of a bud. **Copyright © 2006 John Wiley & Sons, Ltd.**

KEYWORDS: optical tweezers; Raman microspectroscopy; yeast lag phase; G_1 phase

INTRODUCTION

Baker's yeast *Saccharomyces cerevisiae* is an excellent model organism for research in cellular and molecular biology. Since the basic general biochemical mechanisms are highly conserved among all eukaryotes, most current understanding of the cell cycle processes like replication, transcription, translation and protein sorting originally came from experiments performed on yeast.[1] The majority of genes in yeast are homologous to those in humans; hence, molecular biological tools involving yeast are indispensable for the advancement in fields such as anticancer research.[2] Besides being used in the baking industry, yeasts are also important for the production of industrial chemicals and fuels.[3]

Most studies on the metabolism of yeasts as well as their industrial utilisation require their cultivation in liquid media. When a culture of yeast cells is inoculated in a fresh growth medium, they enter a brief lag phase where they are biochemically active but not dividing (Fig. 1(A)). After this lag phase cells enter their cell cycle and start dividing. The cells grow exponentially in number and the population is said to be in an exponential phase of growth. They multiply until some nutrient is exhausted or some metabolite accumulates to toxic concentrations. Subsequently, the cells stop dividing and enter a stationary phase followed by a decreasing phase when cells start dying.[3,4]

The cell cycle of an individual yeast cell can be divided into four phases: [1] M (Mitosis), G_1 (Gap), S (Synthesis) and G_2 (Gap) (Fig. 1(B)). Nuclear division occurs during mitosis. The period after this but before the initiation of nuclear DNA replication, during which the cell monitors its environment and its own size, is known as the G_1 phase. Cells in G_1, if they have not committed themselves to DNA replication, enter G_0, in which the cycle stalls with no S phase. DNA synthesis occurs during the S phase. The period between the completion of nuclear DNA replication and mitosis is termed the G_2 phase. The cell cycle of *S. cerevisiae* is regulated primarily at a point in late G_1 called START. Passage through START is controlled by the availability of nutrients, mating factors and cell size. Buds form just after

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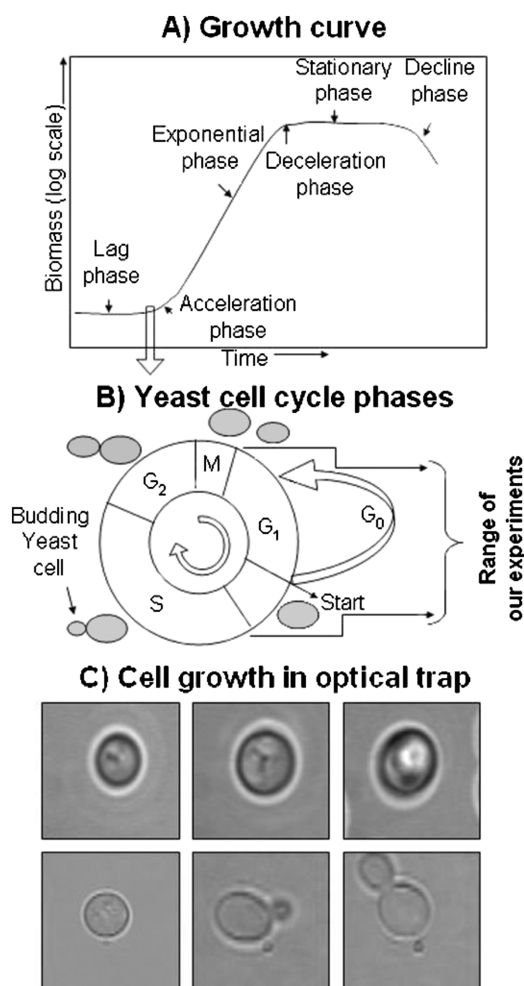

Figure 1. (A) The growth curve shows the phases of yeast cell population. (B) The yeast cell cycle diagram shows the phases of the cell cycle. The abbreviations used for yeast cell cycle stand for the standard terms:[1] M, Mitosis; S, Synthesis; G_1 and G_2, gap phases. For our experiments cells that did not have a bud were chosen. (C) The upper row shows the budding of a yeast cell and its subsequent growth while in the optical trap. The lower row shows the same cell at a moment when it is released from the optical trap.

START and continue growing until they separate from the mother cell after mitosis. Morphological changes undergone by the cell as it enters the cell cycle can be detected under the microscope (Fig. 1(C)).

In industrial applications, yeast is usually propagated in a number of steps before being inoculated into the final fermentation medium. This inoculation culture is often not well defined and hence the physiological condition of the yeast culture may greatly affect the duration and outcome of the fermentation. To understand the physiological condition of the yeast cells in order to optimise these fermentation processes, it is important to understand the biochemical changes during lag phase and growth initiation (G_1 phase).

The biochemical assays and techniques commonly used in molecular biology have limitations. They involve lengthy procedures and cannot be carried out on living cells. Moreover, they give only statistical information for a cell population. Fluorescence microscopy is capable of imaging the distribution of a single molecular species in a cell but requires the use of specific fluorophores. Raman microspectroscopy has been successful for non resonant *in situ* studies of chromosomes[5] and imaging of various cellular components without the use of fluorescent labels.[6] It can monitor kinetic processes in living cells in real time[5–9] and can also detect changes in the biochemical composition of the cell at an earlier stage than fluorescent microscopy can.[10–12]

Optical tweezers[13,14] provide an excellent tool to study nonadherent cells by immobilising them in solution.[15,16] Both Raman microspectroscopy and optical tweezers require a tight focus and a combination of these techniques can be readily achieved. This optical tweezers Raman microspectroscopy (OTRS) technique has been successfully applied for the study of biological cells and nonbiological particles.[17–29] Recently, we have shown that during the fermentation process, the production of glycerol and ethanol can be monitored in real time in a single yeast cell.[8]

In this article, we have applied our dual OTRS system[30] to study the biochemical changes taking place during the lag phase and G_1 phase of a single *S. cerevisiae* yeast cell. We show that Raman microspectroscopy can detect in real-time the adaptation of a single cell to its environment during the lag phase of growth. When the yeast cell was kept in the optical trap for a sufficiently long time, we also detected the bud initiation process during the late G_1 phase of the cell cycle.

EXPERIMENTAL

Sample preparation

S. cerevisiae yeast cells (haploid strain from Euroscarf, BY4741) were grown until the exponential phase in synthetic defined media with complete supplements (SDC) under standard conditions. SDC gives a comparatively low background Raman signal and can provide sufficient nutrients to the cell for the duration of the experiment. Lag phase was achieved by diluting these cells in SDC in the sample holder. The pH of the medium was 5.5. In all Raman measurements, the cells were diluted such that a single cell could be trapped with no other cells in the surrounding medium. The exponential phase was confirmed by measuring the optical density of the culture, which was kept between 0.2 and 0.5. A Pharmacia Biotech UV/visible spectrophotometer (Ultrospec 2000) was used for this purpose at 600-nm wavelength. NaF was purchased from Fluka Biochemika and NaN_3 from Riedel-de Haën.

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Confocal Raman microscope and optical tweezers

Our experimental set-up has been described in detail elsewhere.[8,30] Briefly, a diode laser operating at 785 nm was used for the trapping and excitation of Raman spectra with an average power of about 5 mW at the sample. Cells were trapped inside a temperature-controlled (30 °C) custom-made sample holder with a 100-μm thick fused silica cover slip. The holder was placed on an inverse Olympus IX 51 microscope equipped with a 100x oil immersion objective and a micrometer-controlled x-y stage. The backscattered light is collected by the microscope objective and after a holographic notch filter it passes through a confocal system with a 100-μm pinhole. The spectrometer had a 600 lines/mm grating and was fitted with a thermoelectrically controlled charge-coupled device (CCD), cooled to −100 °C. A CCD camera attached to the microscope provided optical images during experiments. Raman spectra were recorded with a spectral resolution of 8 cm^{-1} over a range of 1100 cm^{-1}, between 570 and 1670 cm^{-1}.

Before measurements were made on living cells the signal to noise ratio of the Raman spectrum was maximised using a trapped polystyrene bead of 4.5 μm. Fluorescence background was removed by fitting a baseline, all the spectra were normalised in area and finally smoothed by applying a Savitsky-Golay filter. Error bars were calculated by taking into account the errors due to usual error sources such as the noise of electronic equipments (shot noise, processing noise and dark current noise)[6] as well as the error specific to our experiment such as Brownian motion of a trapped object.

For time resolved measurements, the initial spectrum $S(T_0)$ at time = 0 was subtracted from each of the subsequent spectra $S(T_t)$ to obtain difference spectra.[8] Plotting the sequential difference spectra makes it easier to visualise small changes in peaks over time without altering the data.

RESULTS AND DISCUSSION

We used a single beam optical trap with a trapping beam size of approximately 0.6 × 0.6 × 1.2 μm. From the point of view of optical properties, the cell consists of areas with different refractive indices. The trapping force is high for the areas with high-refractive indices such as nuclei or small lipid vesicles that occur, naturally, in the cytoplasm.[31,32] In the single beam trap the trapped cell, normally measuring about 5 μm in diameter, continues to move and rotate near high-refractive index areas due to Brownian motion and organelle motility (Fig. 1(C)). Hence results obtained by us cannot give spatially resolved information on biochemical changes and because of the movement of the trapped cell during the acquisition time as mentioned above, we acquired time-averaged Raman signals from the part of the cell with high-refractive indices.

Lag phase

To study the lag phase of growth and G_1 phase of yeast cell cycle in a feasible time-period, we chose to inoculate an exponential phase culture of yeast cells in pure medium inside a temperature-controlled sample holder. The cells were diluted such that a single cell could be trapped. This dilution amounts to a change from the carefully regulated environment the cells were in during the exponential phase and thus brings the cells into a lag phase. After diluting the yeast cells we immediately looked for cells that did not have a bud, meaning that the cell was either in the G_0 or G_1 phase as the bud does not start to grow until the S phase. Each cell was trapped continuously for up to 3 h. The budding of a cell after completion of the measurement indicated that the cell was progressing through the cell-cycle and had not stalled in G_0. Only measurements taken from cells that subsequently budded were considered, in this case 14 cells. As bud initiation was observed to occur after trapping, and the time taken by the cells to initiate a bud inside and outside the trap was similar, this implies that the 785-nm laser beam was not damaging the cell in the G_1 phase. Earlier, we observed that yeast cells cannot sustain even 400 μW of 632.8-nm laser radiation for long periods of time.

The initial spectra taken at various trapping times are shown in Fig. 2(A). Figure 2(B) shows the data processing of a single spectrum, baseline subtraction and smoothing. The initial spectrum of the cell, taken just after diluting the cells in the sample holder and trapping it, was subtracted from subsequent data to get each difference spectrum. Figure 3(A) shows the changes in difference spectra, at 2, 6, 10, 14 and 18 min after trapping, in the initial lag phase of a single trapped yeast cell, with tentative peak assignment as indicated in Refs 33, 34. Numerous peaks are observed after trapping and we assign (see Table 1) the peak at 782 cm^{-1} to RNA, 1450 cm^{-1} mainly to proteins and lipids, the band centred at 660 cm^{-1} to nucleic acids and proteins and another broad band centred at 1150 cm^{-1} to proteins.[34,35] The appearance of these peaks could be related to the lag phase of cell growth while in the optical trap. Peaks are also observed at 610, 1070 and 1370 cm^{-1}. The Raman peaks at 782 and 813 cm^{-1} have previously been assigned to RNA and the Raman peak at 1300 cm^{-1} to lipids.[12]

The ratio of the RNA (813 cm^{-1}) and protein (1005 cm^{-1}) peaks in the Raman spectra of murine embryonic stem cells have previously been proposed as an indicator of mRNA translation during differentiation *in vitro*,[12] and the peak at 1005 cm^{-1} has been used as an indicator of protein denaturation in the cell.[33,36] However, in yeast cells under trapped and non-stressed conditions, the known protein peaks such as 757, 1005 and 1660 cm^{-1} do not show any definite upward or downward trends. As an example, the behaviour of the peak at 757 cm^{-1} with time during the initial lag phase can be seen in Fig. 3(B). Throughout this set of experiments, the 1005 cm^{-1} peak and 757 cm^{-1} peak behave similarly. The Raman peak at 813 cm^{-1} also represents RNA and shows a trend that is similar to the 782 cm^{-1} peak. For this study, we have chosen the latter to represent RNA as it

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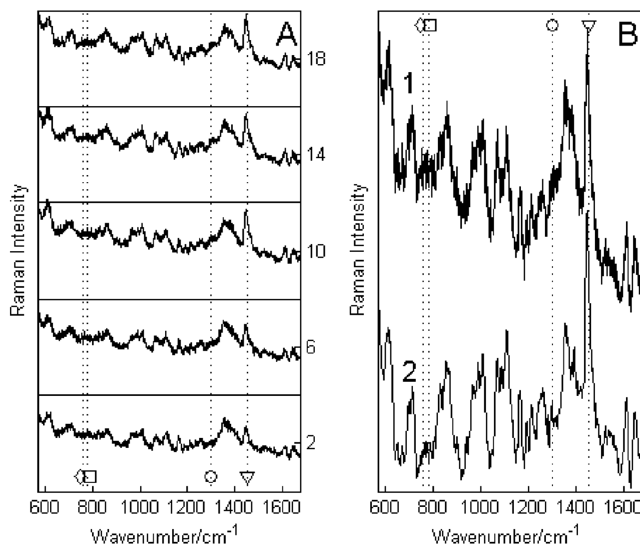

Figure 2. (A) Original data after removal of cosmic rays. (B1) Close up of data at 18 min. (B2) Processing of data B1 (fluorescence background subtracted, normalised and smoothed).

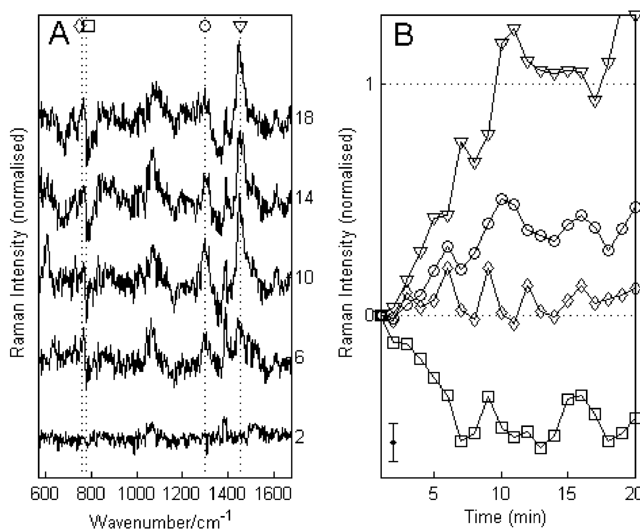

Figure 3. (A) Difference Raman spectra of a yeast cell during lag phase with time elapsed since trapping (indicated at right). (B) Plot of intensities of Raman peaks at 757 [◊], 782 [□], 1300 [○] and 1450 cm^{-1} [Δ] *vs* time during lag phase. Error bar represents a confidence interval [-2σ, $+2\sigma$] corresponding to a probability of 0.95 of finding the signal inside it. The acquisition time was 60 s.

shows more consistent behaviour from cell to cell during the lag phase.

The RNA peak at 782 cm^{-1} is observed to decrease while the lipid peak at 1300 cm^{-1} and the protein and lipid peak at 1450 cm^{-1} increase during the initial lag phase (Fig. 3(B)). Initially, an acquisition time of 180 s was used but later this time was decreased to 60 s as it improved temporal resolution but still had a signal to noise ratio capable of elucidating different cell processes. It was observed that the RNA peak at 782 cm^{-1} decreases only for about 8 min (Fig. 3(B)) and the signal levels off to a small oscillation that is within the tolerance limits defined.

Table 1. Wavenumbers [cm^{-1}] of the Raman peaks observed for a single yeast cell excited at 785 nm and their assignment, according to the references mentioned

Wave number [cm^{-1}]	Assignment[12,15,33]
610	Proteins (Phe)
660 (broad band)	Nucleic acids (T, G) and proteins (C–S str)
757	Proteins (Trp ring br)
782	RNA (U, C, T ring br)
813	RNA (O–P–O str)
1005	Proteins (sym ring br Phe)
1070	Nucleic acids (PO_2^-str)
1150 (broad band)	Proteins (C–C/C–N str)
1300	Lipids (CH_2 twist)
1450	Lipids and proteins (CH def)
1660	Amide I

In one of the protein expression studies performed on a haploid strain of yeast cells,[37] it was mentioned that during an appreciable change in the cellular environment, such as a shift in carbon and energy source, the lag phase can last for hours and the cells reset themselves morphologically as well as physiologically. The main observation was that during the initial lag phase there was an immediate pausing or shutdown in the synthesis of many proteins that occurred just after inoculation. The levels of protein present in the cells subsequently increased as the lag phase progressed. The analyses were performed on cell samples after inoculation at intervals of 10, 20, 40, 60 and 180 min. In two studies, it was noted that the levels of mRNA (messenger RNA) and proteins in yeast cells during the early lag phase did not increase or decrease together.[37,38] However, the cells studied were from different phases of their cell cycle unlike our case, where a single cell was chosen from the G_1 phase of its cell cycle.

If we assign the 782 cm^{-1} peak to mRNA, our results suggest a similar behaviour of proteins and mRNA in the early lag phase stages. As discussed above, since protein synthesis stops just after inoculation we can exclude the possibility of mRNA translation. The decrease observed in this RNA peak in the first 8 min after inoculation may thus be attributed either to the biochemical degradation of mRNA or a change in the conformation of ribosomes already present in the cytoplasm or both.

In accordance with the transcriptional and protein expression analyses mentioned above, we observe that during the initial lag phase the levels of mRNA decrease. The degradation and synthesis of mRNA and proteins and

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changes in the rate of synthesis of rRNA and ribosomal proteins during the lag phase enable the cells to multiply with a rate adapted to the new environmental conditions.[38,39]

The sharp decrease observed in the signal of 782 cm^{-1} peak for the first 8 min, just after inoculation, can be taken as a specific marker for the initial lag phase. This peak does not show a similar behaviour in time for cells trapped in late lag phase (see Control section). The comparison of the behaviour of Raman peaks at 782, 1300 and 1450 cm^{-1} for cells in lag phase and late lag phase may also point to the fact that the cytoskeleton of the cell rearranges itself after inoculation in fresh medium. As the lag phase proceeds, new mRNAs and ribosomes may compensate for this, causing the signal of 782 cm^{-1} peak to level off.

The increase observed in the Raman peaks at 1300 and 1450 cm^{-1}, which is different for the cells trapped in initial lag phase and for the cells trapped in late lag phase, could result from an increasing concentration of proteins and lipids but could also be due to the trapping conditions. This will be discussed further in the Control section where studies on cells trapped in late lag phase and also on dead cells are detailed.

Bud formation

Although many changes can be seen in the spectra during the late lag phase and the G_1 phase, the overlap of Raman peaks for different constituents can make the spectral interpretation difficult. Therefore in each measurement we tried to relate Raman spectral changes to the observed morphological changes, e.g. bud initiation, seen in the cell. For a free cell in glucose minimal medium, the average doubling time[40] of *S. cerevisiae* is 120 min at 30 °C. The growth of yeast cells in the optical trap, after the bud initiation, is observed to be comparatively slow. We estimate that in the optical trap it will take approximately 3 h for a cell to complete its cell cycle.

Figure 4(A) shows the changes in difference spectra, at 54, 60, 66, 72 and 78 min after trapping, of the same cell in late G_1 phase or early S phase. The peak at 1450 cm^{-1} that represents lipids and proteins grows considerably. During the budding process, many proteins are synthesised and lipid vesicles carry cargo to the budding site. We believe that the rise of this peak is an indicator of this phenomenon. It is known that small granules are attracted towards the laser trap in a living cell. However, because of the presence of the cytoskeleton, they cannot move freely.[32] At low power, as in our experiments, we suppose that small clusters of proteins and lipids not embedded in the cytoskeleton of the living cell can move freely and contribute to the observed increase in the Raman spectrum.

Another interesting peak was observed at 1602 cm^{-1}. Earlier it was proposed[11] that the intensity of this peak is a measure of the respiratory activity in a mitochondrion of the fission yeast *Schizosaccharomyces pombe* because it decreased after the addition of potassium cyanide (KCN) to the cell medium. In Ref. 41, the mitosis of *S. pombe* was studied and the same peak was observed during the late G_1 phase of the cell cycle. It was proposed that mitochondria were located at the central part of the cell during this time. Since our observations with *S. cerevisiae* show that the 1602 cm^{-1} peak is observed during the late G_1 phase when budding starts, and this peak is also observed in Ref. 41 when *S. pombe* cells are going to divide, we suggest that this spectral feature is related to the cascade of events during the fission of *S. pombe* and the budding of *S. cerevisiae*. Also in agreement with Ref. 41, we observed that the rise in 1602 cm^{-1} peak is accompanied by a rise in the lipid peaks at 1300 and 1450 cm^{-1} (Fig. 4(B)).

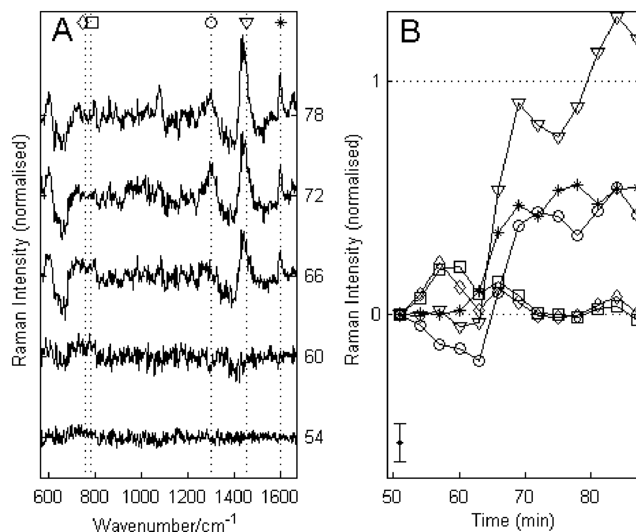

Figure 4. (A) Difference Raman spectra of a yeast cell during late G_1 phase with time elapsed since trapping (indicated at right). (B) Plot of intensities of Raman peaks at 757 [◊], 782 [□], 1300 [○], 1450 [△] and 1602 cm^{-1} [*] *vs* time during late G_1 phase. Error bar represents a confidence interval [-2σ, $+2\sigma$] corresponding to a probability of 0.95 of finding the signal inside it. The acquisition time was 180 s.

Control

The behaviour of a given yeast cell is dependent on two factors, namely, the genetic constitution and environmental conditions. For our experiments, we took cells of the same genetic constitution but changed their environment. We observed sharp changes in the Raman spectra of a cell trapped immediately after dilution. This can be taken as the initial lag phase of the cell. In order to study a cell that had already passed its lag phase, we performed our experiments 20 min after dilution. This acted as a control experiment. The changes in the Raman peaks at 782 and 1450 cm^{-1} were not the same as observed for the cells that were in the lag phase (Fig. 5).

To confirm whether the observed time behaviour of the peaks at 782, 1300 and 1450 cm^{-1} was a result of the biochemical changes inside the cell or of trapping, we performed a study on yeast cells killed either by boiling

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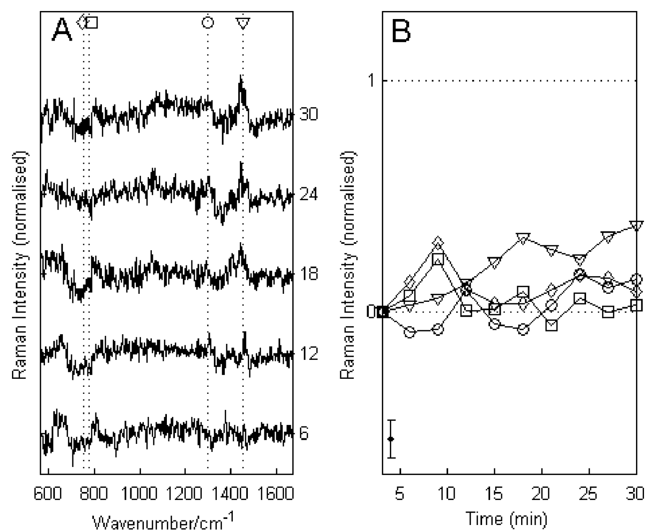

Figure 5. (A) Difference Raman spectra of a yeast cell during late lag phase with time elapsed since trapping (indicated at right). (B) Plot of intensities of Raman peaks at 757 [◊], 782 [□], 1300 [○], and 1450 cm^{-1} [Δ] *vs* time. Error bar represents a confidence interval [-2σ, $+2\sigma$] corresponding to a probability of 0.95 of finding the signal inside it. The acquisition time was 180 s.

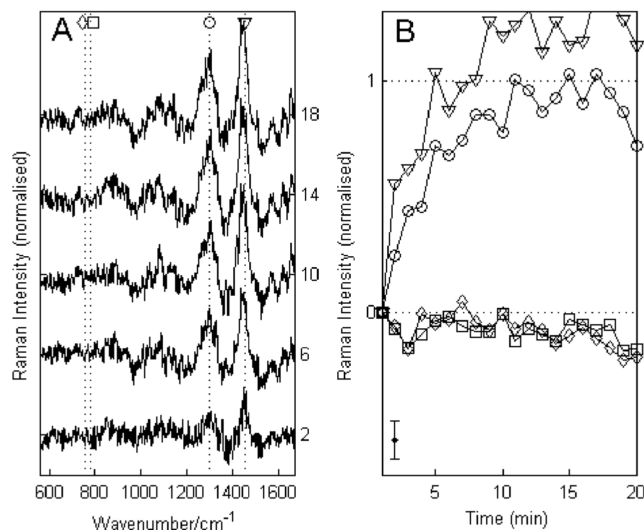

Figure 6. (A) Difference Raman spectra of a dead (chemically treated) yeast cell with time elapsed since trapping (indicated at right). (B) Plot of intensities of Raman peaks at 757 [◊], 782 [□], 1300 [○] and 1450 cm^{-1} [Δ] *vs* time. Error bar represents a confidence interval [-2σ, $+2\sigma$] corresponding to a probability of 0.95 of finding the signal inside it. The acquisition time was 60 s.

or by a chemical protocol. For chemical treatment, NaF (sodium fluoride) and NaN_3 (sodium azide) were added to the culture to final concentrations of 10 mM each. This treatment resulted in cell death. Sodium azide specifically inhibits the mitochondrial activity, whereas one of the main actions of the fluoride ion is to inhibit the enolase enzyme of the Embden–Meyerhof pathway.[42] Fig. 6(A) shows that the peaks at 1300 and 1450 cm^{-1} increase but there is no effect on RNA peak at 782 cm^{-1}. Also, the increment in the peaks at 1300 and 1450 cm^{-1} occurs only for about 10 min (Fig. 6(B)) and after that the intensity of the peaks becomes fairly constant. This is quite a different behaviour from the changes seen in these peaks in the lag phase (Fig. 3(B)) or in the late G_1 phase (Fig. 4(B)). A similar behaviour of the 782, 1300 and 1450 cm^{-1} peaks of boiled cells was observed as those of chemically treated cells but in the former case the protein peak at 1005 cm^{-1} is stronger and the cells appear more opaque. Keeping in mind that in the dead cell the cytoskeleton is not functioning as normal, the proteins and lipids may be attracted to the focus of the trapping laser. In the cells trapped in late lag phase (Fig. 5(B)), this increment in protein and lipid peaks was not observed, which may point to the fact that after inoculation the cell rearranges its cytoskeleton during the lag phase. This may be because of the possible hyperosmotic or hypoosmotic changes encountered by the cell in its micro-environment after inoculation in a fresh medium.

When experiments were performed on the cells kept in original medium, i.e. taken directly from an exponentially growing culture and were not diluted in a fresh medium, the Raman peaks mentioned above were observed to fluctuate without showing a definite trend for the first 20 min of trapping.

A separate control was performed by diluting the cells in supernatant left after pelleting down the cells taken from the exponential phase of growth. This will not yield the same environmental change as diluting the cells in fresh medium. The Raman peaks mentioned above also did not show a consistent behaviour for the first 20 min of trapping a cell. Thus the possibility that in our case the changes seen were caused by other environmental factors, and not by inducing the lag phase by the dilution of the cells in a fresh medium, can be ruled out.

CONCLUSIONS

Using Raman microspectroscopy together with optical tweezers, we monitored real-time changes known to occur during the lag phase in a single yeast cell. Using favourable trapping and Raman excitation conditions, the decrease in mRNA levels and synthesis of lipids and proteins in the lag phase could be tracked over time. During bud growth, synthesis of lipids and proteins was also detected. This shows the ability of this technique to detect real-time biochemical changes in a single cell and the potential for elucidating the biochemical processes in living cells at a single cell level. The most obvious application lies in studying the effect of drugs and toxins on the biochemistry of living cells. However, it is difficult to trap a budding yeast cell, as it becomes asymmetrical, in a single beam trap. A multi-beam tweezers

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set-up is needed for better control of trapping forces over the cell and it could also facilitate the study of mammalian cells, which are much larger compared to our yeast cells. A method for doing this is currently under development in our lab.[43]

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