Eur Biophys J (2008) 38:111–119
DOI 10.1007/s00249-008-0359-z

BIOPHYSICS LETTER

Metabolic activity interferometer: description and calibration of an interferometric method to measure growth of mycobacteria

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Received: 17 October 2007 / Revised: 23 April 2008 / Accepted: 3 July 2008 / Published online: 26 July 2008

Abstract An interferometer that measures the refractive index changes due to bacterial metabolism is described. The apparatus permits simultaneous and real time measurement of bacterial growth in several samples of slowly growing mycobacteria. The error sources are discussed and the sensitivity of the apparatus is tested. For the species *Mycobacterium bovis* BCG and *M. smegmatis*, a relation between refractive index change and bacterial concentration is determined experimentally and the time constant of bacterial growth is measured.

Keywords Bacterial growth · Tuberculosis · Interferometry · Refractive index

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Introduction

During the recent decades, resurgence of infectious diseases caused by mycobacteria, such as tuberculosis (TB), has occurred especially due to epidemic HIV/AIDS, which greatly increases the risks of latent infections or re-infection progressing to active disease (Pfyffer and Vincent 2005; Montoro and Rodriguez 2007). Furthermore, spontaneous mutation associated with inadequate courses of anti-tuberculosis therapy has led to the emergence and spread of drug-resistant and multidrug-resistant strains. The percentage of multidrug-resistance is as high as 10% in tuberculosis HIV/AIDS patients (Frieden et al. 2003; Chan and Iseman 2002). Patients infected by a strain that is resistant to at least isoniazid and rifampicin have enhanced mortality and need to be treated by more expensive strategies such as directly observed therapy (Montoro and Rodriguez 2007; Frieden et al. 2003).

Mycobacteria from the complex *Mycobacterium tuberculosis* are slowly growing bacteria that take about 30 days to form visible colonies in solid culture medium (Löwenstein–Jensen) and about 15–20 days in solid culture medium Middlebrook 7H10 supplemented with Oleic acid Albumin Dextrose Catalase (OADC) (Pfyffer and Vincent 2005). Occasionally visible colonies may appear after only 8 weeks. Therefore, the mere isolation of *M. tuberculosis* may take 2 months time and meanwhile the patient may receive an inadequate treatment in case of resistant bacteria. A subsequent test whether the bacterial strain is resistant against antibiotics requires creation of a stock of bacteria so that several assays can be prepared. The time required depends crucially on the sensitivity of the testing methods because this factor determines the size of the necessary initial stock.

Early detection of drug resistance constitutes one of the priorities of TB control programs, allowing the appropriate treatment of the patient and surveillance of drug resistance (Martin 2007). Detection of drug resistance has been made by intensively laborious and long-time conventional methods, such as the agar proportion method. In this method, dilutions of the inoculums are spread onto agar-based medium containing drugs, and the numbers of colony forming units (CFU) are compared with the ones growing on drug-free medium. The conventional determination of susceptibility of *M. tuberculosis* against antibiotics takes typically 1–3 months time (Clinical and Laboratory Standards Institute 2003). The disadvantages of the conventional methods have stimulated the development of new approaches. The keystone of any rapid isolation and susceptibility testing method is a sensitive and precise method to measure bacterial growth. The following methods measure bacterial growth by evaluating the chemical throughput of mycobacterial metabolism: the BACTEC 460 TB measures the CO_2 production using ^{14}C marked nutrients, BACTEC MGIT 960 uses fluorescence measurements of oxygen usage and BACTEC 9240/9120 uses fluorescence measurements of CO_2 production. These methods permit handling many probes in a safe and automatic way, and are very sensitive. In the present work an alternative equally sensitive method of measuring the chemical throughput is described and tested that permits measuring bacterial growth quantitatively and in real time. The measurement is nondestructive, i.e. the measured samples can be used as bacterial stock for future tests.

In previous work, it had been shown that bacterial metabolic activity can be monitored by very precise interferometric measurements of refractive index changes of the culture medium (Jardim et al. 2003). But the relation between index change and bacterial concentration had not been determined. Moreover, the apparatus was totally inadequate for biomedical measurements. The main restriction was that only one sample could be measured at a time.

In the present work a prototype of an apparatus that monitors mycobacterial growth interferometrically and that permits measurement of several samples simultaneously in real time is described. The aim of this work is to show the viability of the apparatus for mycobacterial growth detection, to characterize the errors involved and to establish a relation between the rate of refractive index change ($\Delta\dot{n}$) and mycobacterial concentration. To show that the method is appropriate for growth detection of slowly growing mycobacteria such as *M. tuberculosis* the species *M. bovis* BCG (Bacillus Calmett–Guérin) was used as a model. The rapidly-growing *M. smegmatis* was also tested for comparison. The relation between refractive index change and mycobacterial population and the time constant of bacterial growth was determined for these species.

Description of the apparatus

The apparatus consists of an interferometer that permits monitoring the time evolution of the difference of refractive index of two liquids in several samples. In the applications shown here the liquids are culture media, one of which contains mycobacteria. In general both liquids suffer some index change due to oxidation; however, the liquid containing bacteria exhibits an additional index change due to bacterial metabolism. Provided both liquids have equal chemical composition, the rate of change of the difference of refractive index is a measure of the total metabolic activity that goes on in the sample. Therefore, the apparatus received the name metabolic activity interferometer (MAI).

The task was to build an apparatus that measures several samples with such high sensitivity that the detection limit was comparable with other techniques. There are two ways of interferometrically monitoring refractive index changes in several samples: multiplying the optics or exchanging samples by means of relative motion between samples and optics. The first alternative involves less problems concerning stability but it leads to extremely expensive constructions. Therefore, we decided to cope with the stability problems that are necessarily involved in the second alternative.

In the apparatus described here the samples are fixed on a rotating stainless steel disk that moves them into a Michelson interferometer, (Heavens and Ditchburn 1991) where they get measured every 20 min. The sample holders, which are made of stainless steel (316), have two adjacent channels of equal length (37 mm) that terminate on both sides in glass windows so that the light beams of the two interferometer arms can be sent through the channels, as shown in Fig. 1. The input beam is an expanded He–Ne laser beam ($\lambda = 0.6328$ μm). Each of the two channels contains 1 mL of the culture medium and an air stock of 3.1 mL, but only one of them contains bacteria. This way the metabolic activity of the bacteria creates relative changes of refractive index of the culture medium in the two channels and that provokes relative phase changes of the interfering light beams.

A precise determination of the relative phase of the interfering beams would be rather difficult with just a single intensity measurement. Better results can be obtained by modulating the phases. This can be done with the help of Pockels cells (Heavens and Ditchburn 1991) or by moving one of the mirrors M1 or M2 with a piezoelectric drive. This introduces an additional parameter x, for example, the electric field of the Pockels cell. The light intensity of the recombined beams is then an oscillating function of x. This function permits a precise determination of the relative phase. For the present apparatus the choice

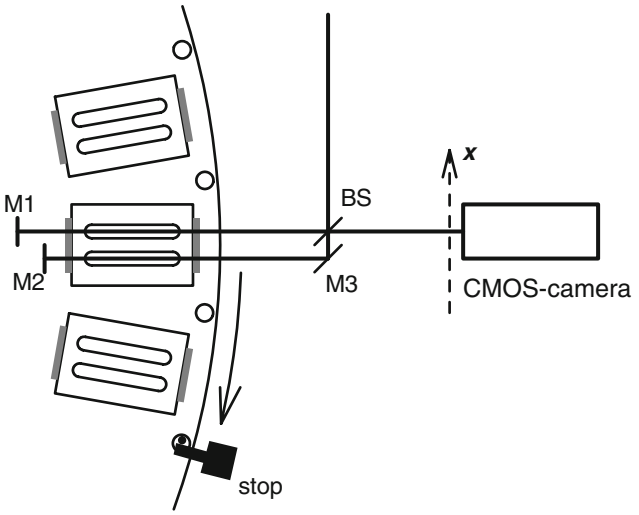

Fig. 1 Schematic geometry of the essential parts of the MAI. A beam splitter BS and three mirrors M1-3 form a Michelson interferometer. The light beams of the two interferometer arms pass through a sample holder that is fixed on a rotating disk. The *dotted arrow* in front of the CMOS-camera indicates a spatial coordinate axis that is used during phase detection. The stop permits precise positioning of the sample

of the extra parameter x was determined by the aim of building an inexpensive apparatus. One of the mirrors M1 or M2 was simply tilted so as to obtain vertical interference fringes. In this way the extra parameter x is the spatial horizontal coordinate perpendicular to the outgoing beam. The oscillating intensity function $I(x)$ is captured by a commercial complementary metal-oxide-semiconductor-camera (CMOS-camera). The camera lens was taken off and the red pixel signals were summed over vertical columns. This summing eliminates the vertical coordinate and improves the signal-to-noise ratio. The signal analysis is performed by a personal computer in real time. The program subtracts the mean value, normalizes the amplitude, and finally fits a trigonometric function. The relative phase of the light beams is then determined from the argument of that function. Figure 2 shows an example of a picture, a normalized intensity curve and the trigonometric fitting. The measured phase is of course only determined modulo an integer number of fringes. The computer program adds appropriate integer values so that the phase becomes a continuous function of time. This procedure may lead to wrong results when the bacterial activity is so high that the interference fringes move more than one fringe during one turn of the sample disc. However, such high activities are not typical with slowly growing mycobacteria. Typical values are of the order of 10^{-3}–10^{-1} fringes per turn.

The refractive index changes are of the order of 10^{-7}–10^{-5} per day and one observes them during periods of time as long as 3–40 days (Jardim et al. 2003). Such small changes and long observation times require extremely stable conditions.

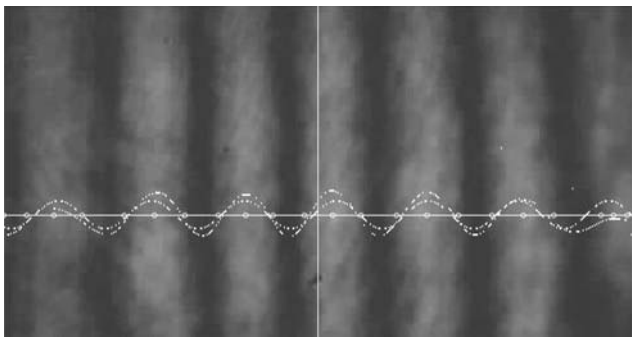

Fig. 2 Fringe images together with a graph of averaged light intensity and trigonometric fitting (in arbitrary units). The *horizontal line* is the spatial coordinate axis x, which is indicated in Fig. 1 in front of the CMOS-camera. This coordinate is identical with the x-axis of the graph. The fringe image corresponds to a single instant and gives one measurement point for a sample. If there were metabolic activity in that sample the following image of the same sample would have the fringes at a slightly different place

Mechanical stability of the interferometer itself is guaranteed by a compact geometry of the interferometer. The whole apparatus is positioned on a 30 mm stone plate of 55 cm × 80 cm that rests on a 30 mm layer of foam on an ordinary table plate. The compact form of the Michelson interferometer is so stable that strong beating on the table plate does not provoke any noticeable jitter of the fringe images.

Another critical point of mechanical stability is the reproducibility of sample positioning. The sample holder geometry has small imperfections. The sample windows are cut from ordinary float glass and are glued onto the sample holder faces. This gives only a limited uniformity of optical path lengths in directions perpendicular to the light beams. Therefore, it is important to position the samples always at the same spot. The sample disc, which rotates on tight ball bearings, has 8 mm holes near the border. Whenever a sample moves into the interferometer a hardened steel bolt is stuck through one of the holes and the motor that drives the sample disc presses the disc against that bolt, which in turn is pressed against a fork like stop. During capture of the fringe image the motor is turned off. The motor is a step motor coupled to a toothed gearing and a worm-drive, an elastic clutch as well as a slipping clutch. The latter is only used to move the disk by hand when the samples are positioned on the disc.

The interferometer and the sample disc are mounted in an aluminum box of 12 mm thick walls. The temperature of this box is kept constant at about 37°C. The constancy of temperature is 1×10^{-3} K. Temperature is measured with a platinum resister whose resistance is monitored with a four wire ohm meter. Heating is effectuated by a fine copper wire densely and uniformly glued on the outer surface of the aluminum box just beneath an outer thermal

insulation. The heating power is regulated with a pulse width modulation scheme in order to keep energy consumption low. This is important because the whole apparatus is supplied with electric energy by means of an uninterruptible power supply.

The laser and the CMOS-camera are mounted on the outer bottom surface of the aluminum box and outside the thermal insulation. Unfortunately it turned out that this configuration caused a considerable dependence of phase measurement on the outer room temperature. Probably this error is due to laser misalignment. We managed to get rid of this error by a subtraction scheme. Originally the apparatus was constructed to measure 20 samples but now we leave one sample place empty and subtract the phase value of that dummy measurement from the sample measurements. This eliminates the problem in a surprisingly efficient way. However, future constructions should take care of the temperature problem in a clearer way.

Besides mechanical and thermal stability the interferometric method of measuring bacterial growth requires uniformity of the physical and chemical composition of the samples as well as absence of biological contaminations. The sample holders, previously sterilized, are filled with their contents in a laminar flow hood. During this procedure the temperature of the sample holders is kept at approximately 37°C in order to avoid future thermal expansion of the air stock in the sample. The samples are sealed up with sticky tape in order to prevent contamination and evaporation of water from the culture medium. Thermal expansion of the air inside the sample holder would break the seal. The samples are transported to the MAI and placed on the sample disc.

Figure 3 shows the interior of the apparatus. The laser, the CMOS-camera, and the motor are located beneath the aluminum box and are not visible. The interferometer can be seen. It rests on two steel columns and bridges over the row of sample holders.

The primary quantity that is furnished by the MAI is a relative phase φ (measured in radians) of the two light beams as a function of time. Apart from an irrelevant constant, this phase is proportional to the difference of refractive index of the liquids in the two channels of the sample holder:

$$\Delta n(t) = n_{\mathrm{ref}}(t) - n(t) = \frac{\lambda}{2L}\frac{\phi(t)}{2\pi} + \text{const.} \qquad (1)$$

L is the length of the sample holder, λ is the laser wave length and t is the time coordinate. n_{ref} and n are the refractive indices of the reference channel and the bacteria containing channel, respectively. It might seem more natural to define Δn as the index of the bacteria containing channel minus the index of the reference channel, but it has been decided to use $n_{\mathrm{ref}} - n$ because it is perturbing to

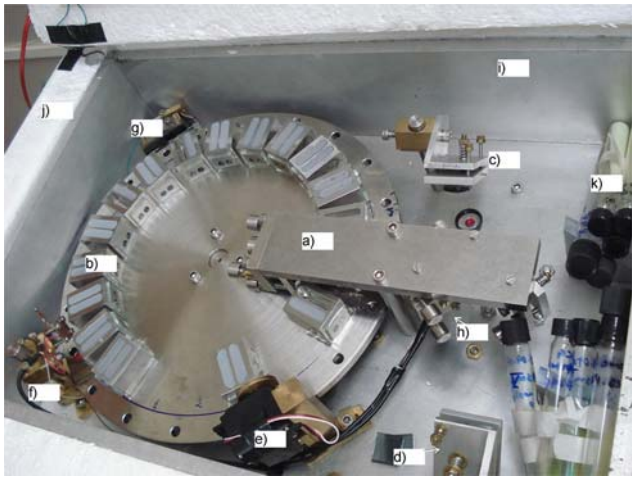

Fig. 3 Photograph of the interior of the MAI taken just before closing the apparatus. *a* Michelson interferometer, *b* sample holders on rotating disc, *c* light input mirror, *d* image exit mirror, *e* precision positioning system with stop and bolt, *f* relative position sensor that triggers the positioning bolt, *g* absolute position sensor that detects sample number 1, *h* thermometer, *i* isothermal box, *j* thermal insulation, *k* stock of bacteria for future experiments

associate bacterial activity and growth with falling curves. The undetermined constant does not perturb. In fact the biologically relevant quantity is the time derivative of Δn. This time derivative $\Delta \dot{n}$ is proportional to the rate at which nutrients are consumed and therefore is a measure of the total metabolic activity in the sample holder. $\Delta \dot{n}$ is calculated from a polynomial or exponential fit of the experimentally measured curve $\Delta n(t)$.

Materials and methods

The present work reports on four experiments that were performed with the MAI with *M. bovis* BCG-*Monroe* and *M. smegmatis*. Table 1 gives an overview of the relevant parameters of the experiments. The total number of samples was higher than indicated in Table 1: the experiments used also control samples without bacteria, some samples were used to evaluate index change of culture medium and some bacteria containing samples had to be discarded because of broken seals.

In the Experiments I–III the bacteria were kept on solid culture medium (Lowenstein-Jensen) at a temperature of 37°C. Twenty-eight days before every experiment, colonies of *M. bovis* BCG were taken with a sterile wooden stick, separated without liquids with the help of glass beads in a shaking screw-cap tube on a vortex mixer during 1–2 min, suspended in sterile Phosphate Buffered Saline (PBS), and finally spread on the surface of sterile solid culture medium and incubated at 37°C. This prior replication of bacteria was performed in order to load the

Table 1 Overview of experiments

Experiment	Mycobacteria	Initial concentration (B/mL)	Culture medium	Number of samples evaluated
I	BCG *Monroe*	$10^{3.7\pm0.3}$	7H9 + OADC	4
II	BCG *Monroe*	$10^{3.6\pm1.6}$	7H9 + OADC	12
III	BCG *Monroe*	$10^{4.7\pm0.2}$	7H9 + OADC	12
IVa	*M. smegmatis*	$10^{4\pm1}$	7H9 + ADC	3
IVb	*M. smegmatis*	$10^{4\pm1}$	MMH	3

Initial bacterial concentration expressed in terms of B/mL (bacteria per milliliter). For the values of Table 1 the difference between Colony Forming Units (CFU) and Individual Bacterial Counts (IBC) are smaller than the errors and both units are called B. Culture media: Middlebrook 7H9 broth OADC/ADC enrichment, and modified Mueller Hinton (MMH)

sample holders with viable bacteria in the log phase. The day the sample holders were filled with their contents, a sample of bacteria was taken with a sterile wooden stick, the colonies were smashed with shaking glass beads, and the bacteria were suspended in sterile saline solution increasing the factor of dilution reaching a final opacity of 0.5 in the McFarland scale (1.5×10^8 CFU/mL). This primary suspension was then diluted in sterile PBS in several steps so as to obtain the desired concentration of bacteria in the suspension that was later added to the channel content. However, in the last step of dilution PBS was not used, but the culture medium Middlebrook 7H9 broth (BD-lot.2112134-USA) with Middlebrook OADC growth supplement enrichment. Thereafter, the channels of sample holders previously sterilized in 120°C water vapor for 30 min in autoclave, were filled with culture medium (1 mL in both channels for reference samples and 1×0.9 mL for bacteria samples). 100 μL of the final suspension of bacteria in culture medium was added to 900 μL of culture medium that had been released into the sample channel beforehand. Thereafter the sample holders were closed with sticky tape. The filled sample holders were transported to the MAI, located on the sample disk, and fixed with hot glue. The apparatus was closed and the control program of the MAI was started.

A relation between rate of index change ($\Delta\dot{n}$) and mycobacterial concentration for the species *M. bovis* BCG in 7H9-OADC culture medium was established with 21 samples of the Experiments I, II and III. The concentration C and $\Delta\dot{n}$ were determined at the end of the experiments. $\Delta\dot{n}$ was calculated from fitted curves that describe the final part of the experimentally determined curves $\Delta n(t)$ To measure the final concentrations of mycobacteria in four samples of the Experiment I and five samples[1] of Experiment II the channel content was diluted in ultra pure distilled water to a final volume of 10.0 mL and this suspension was measured in a flow cytometer (Bentley—IBC). The cytometer used actually only 7.3 mL. The remaining suspension was completed with another 6.0 mL of ultra pure water and the resulting higher dilution was again measured with the cytometer. This allowed evaluation of the precision of the cytometer data. Some samples showed considerable errors. These errors may be explained by the fact that the bacteria in aqueous suspension, which is sucked by the cytometer, have the form of colonies and the stirring that is performed to homogenize the suspension just before sucking it into the apparatus is not sufficient to guarantee uniformity. In Experiment III the final concentrations were measured with the flow cytometer diluting always to a final volume of 8 mL. In this experiment four groups of samples were used: the first group was taken out of the interferometer at $t = 5.13$d (where d = day), the second group at $t = 7.05$d, the third at $t = 9.09$d and the final group at $t = 12.00$d. Each group contained three samples.

For three samples of the Experiment I, and six samples of Experiment III it was also possible to use initial concentration and initial $\Delta\dot{n}-$data. The initial concentrations of mycobacteria in the Experiments I–III were determined with four different methods:

a) The suspension was prepared diluting a suspension with known opacity, which was determined with the help of a McFarland scale by visual and photometric comparison. These values shall refer to as the McFarland values.
b) At the end of the Experiment I and II the colonies that were present in the channel containing bacteria were counted. In special cases very big colonies with an aspect of an agglomeration of colonies were counted with estimated weighting factors. Counts done by two members of the team resulted in surprisingly close values (in a total of ten counts typical discrepancies from the mean value were of the order of 12% and the largest discrepancy ever seen was 27%). The number of colonies found in the channel should be equal to the initial number of viable bacteria in that channel.
c) 25 μl of the initial suspension was spread on a solid culture medium (Middlebrook 7H10-agar enriched

[1] The usage of seven samples of Experiment II was limited to the determination of the time constant because of a technical problem of the flow cytometer.

with OADC) and colonies formed during 12 days were counted.

d) In Experiment III the same procedure of sample preparation was used to prepare four tubes of 8 mL with the same bacteria concentration as the sample holders. Theses probes were measured with the flow cytometer. This does not allow judgment of the individual samples but gives an estimate of the mean value of the initial concentration of the samples of experiment III.

The McFarland values of the samples of the Experiment I were approximately two times larger than the values obtained from colonies in the channels. This can be considered a small discrepancy: on one hand colony counting is fairly uncertain and on other hand the opacity of a bacteria containing suspension may also be caused by dead bacteria. The values of initial concentration of the three samples considered in the Experiment I was taken to be the arithmetic mean of values obtained with method (a) and (b). The method (c) was not used in the Experiment I. In the Experiment II the colony counting in solid medium (method c) gave results slightly smaller than the colony counting in the channels ($CFU_{solid}/CFU_{channel} = 0.76 \pm 0.10$), but the colony values from the channels were smaller than the McFarland values by a factor 42.

The initial values of concentration were related to initial values of $\Delta\dot{n}$. These latter values were obtained by extrapolating exponential fittings backwards to $t = 0$. This extrapolation was only possible with reasonable reliability for three samples of the Experiment I and for six samples of experiment III. The extrapolation was done with different fitting intervals and only curves that gave reasonably interval independent results were accepted. The backward extrapolations of Δn curves with the Experiment II were uncertain. This together with the large discrepancy between McFarland values and colony counts made us exclude the initial values of Experiment II from the final results.

The preparation procedure for samples of *M. smegmatis* was the same used in the experiments I–III, but instead of OADC enrichment Albumin Dextrose Catalase (ADC) (Remel lot. 558111 USA) was used. Some samples were also prepared with Modified Mueller–Hinton culture medium (M. H.-Broth with sodium piruvate). But bacterial concentrations were determined only by means of the McFarland scale. *M. smegmatis* do not form heterogeneous populations of colonies but give homogeneous opaque suspensions directly. This permits use of the McFarland scale at the end of an experiment provided the experiment reaches concentrations as higher as 1.5×10^8 B/mL. As the concentration values were based on a single method the estimated error of the logarithm of concentration was established equal 1; $\delta\log(C \times \text{mL/B}) = 1$. The final values of $\Delta\dot{n}$ had to be determined also by an extrapolation of exponential fittings because *M. smegmatis* is so rapidly growing that during the final part of the experiment phase changes during one turn of the sample disk were larger than one fringe. The estimated errors of $\log(\Delta\dot{n} \times \text{d})$ was 0.7. Altogether, the experiments with *M. smegmatis* gave quantitative data of much less precision than the BCG experiments.

The absence of biological contaminations in the sample holders was tested with several methods: (1) all sample holders were examined visually at the moment of removal from the interferometer. (2) In experiments that used cytometer measurements the content of the reference channels were also measured to test whether the counts were compatible with counts in sterile distilled water. (3) For several samples the channel contents were spread on agar medium (Middlebrook 7H10 supplemented with OADC), incubated at 37°C and examined periodically. So far a total of eight experiments have been performed with the apparatus with 116 samples (including control samples) and contamination has been found in two samples, which also showed unusual interferometric curves.

Results

The curves of Fig. 4 show examples of three measurement results of individual samples. The curves correspond to: (a) a sample of BCG *Monroe* (Experiment I) in 7H9 culture medium and OADC enrichment with an initial concentration of viable bacteria of $C(0)=10^{3.7\pm0.4}$ B/mL, (b) a sample of *M. smegmatis* in 7H9 with ADC enrichment (Experiment IV) and (c) a control sample with culture medium without bacteria. The mycobacteria containing samples show an exponentially growing behavior.

The control samples do not always result in absolutely horizontal curves. Typically one finds time derivatives of control curves of the order of 1×10^{-7}/d. The oxidation of culture medium causes index changes of -7×10^{-6}/d. That means the time derivative of a typical control curve is a few percent of the natural degradation of culture medium. The lengths of the channels of the sample holders are equal with precision better than 10^{-3}. So the error source is not related to geometry but is more likely related to unequal chemical composition and/or volume of channel contents. Even though, after 10 days the slowly growing BCG signal, which started with $10^{3.7\pm0.4}$ bacteria, exhibits a time derivative that is undoubtedly bigger than a typical control value. Ten days is also the typical detection time in BACTEC systems.

In general, the exponentially growing curves show still another kind of error, which limits the precision of quantitative evaluations. Our phase detection system has

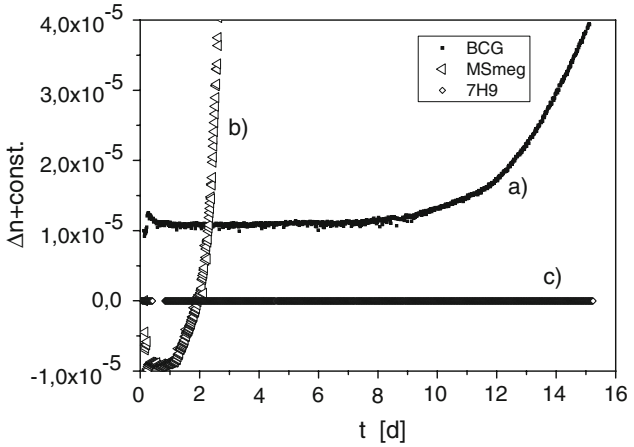

Fig. 4 Experimental results of three samples showing refractive index change as a function of time. *a* BCG Monroe in 7H9 + OADC with initial concentration of bacteria $C(0) = 10^{3.7\pm0.4}$B/mL (5,000 bacteria per milliliter), *b* a sample of *Mycobacterium smegmatis* in 7H9 with ADC enrichment with an initial concentration 1.5×10^4B/mL, and *c* a control sample of culture medium without bacteria

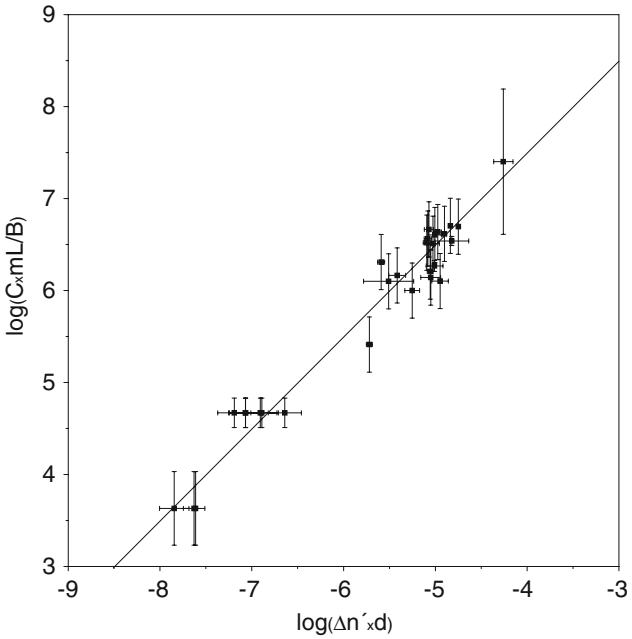

Fig. 5 Log-Log representation of the relation between bacterial concentration and rate of index change for BCG. The results are shown in unit independent form: Abscissa: logarithm of the time derivative of index difference times day $(\log(\Delta n' \times \mathrm{d}))$, Ordinate: logarithm of bacterial concentration times milliliter per bacteria $\log(\mathrm{C} \times \mathrm{mL/B})$. The *straight line* has slope 1. Data points in the *upper right region* stem from values taken from the final part of experiments and nine data points to the *left* of $\log(\Delta \dot{n} \times \mathrm{d}) = -6.5$ represent initial values

typically errors of the order of 2% of a fringe. This corresponds to an error in refractive index of 2×10^{-7}. As long as these errors are purely statistical they do not perturb because the number of measurement points is large. One has about 170 measurement points in the time interval of one typical time constant of bacterial growth. But there is also a systematic phase error $\delta\phi$ of the same order that shows some sort of almost periodic dependence on ϕ. Future constructions should eliminate this sort of oscillatory error using better procedures for phase detection.

In some samples strange deviations from the exponential behavior can be seen during the first 20 h of the experiments, as can be seen with the examples of Fig. 4. This unexpected behavior is due to thermal perturbations during loading of the apparatus with sample holders. The corresponding data points have to be ignored. In future constructions the apparatus should be integrated into a laminar flow hood and the samples should enter the interferometer box through a thermal air lock, so as to generate valid data right from the beginning.

As long as the experiments are performed in such a way that depletion of nutrients and oxygen can be neglected one expects bacterial concentration and time derivative of the refractive index difference to be proportional. In order to show that such a linear relation between interferometric signal and concentration does exist we correlated the quantities $\mathrm{d}\Delta n/\mathrm{d}t$ and C in a bi-logarithmic diagram. The logarithmic analysis is more appropriated in this case for two reasons: first the values of concentration vary almost four orders of magnitude and second the errors of concentration values are large and of multiplicative character. So a measured bacterial concentration may for instance be too large or too small by a factor 2. Figure 5 shows the relation between bacterial concentration and rate of index change for the case of BCG in 7H9-OADC in a logarithmic representation. The upper group of data points stems from final values and nine data points to the left of $\log(\Delta \dot{n} \times \mathrm{d}) = -6.5$ represent initial values. A linear fit of data points, $y = a + bx$, without errors as weight[2] results in $a = 11.41 \pm 0.23$ and $b = 0.986 \pm 0.040$, with a correlation coefficient $R = 0.97878$ and standard deviation SD = 0.22. The deviation of b from one is smaller than the error (one standard deviation) and one may assume a linear relation between C and $\Delta \dot{n}$. If one fixes $b = 1$ and performs a minimum square fit one obtains

$$a = 11.49 \pm 0.14. \quad (2)$$

In equation (2) the error (error of the mean value) corresponds to 3 standard deviations. With this result one may state that for BCG in 7H9 with OADC enrichment, the bacterial concentration and the interferometric signal $\mathrm{d}\Delta n/\mathrm{d}t$ are proportional and the constant of proportionality is

[2] The error bars are estimated based on comparisons of different analysis of any bit of data. But a careful statistical analysis showed that they are in fact inconsistent because many data points would be very unlikely. Some error bars had been estimated too small.

$$\frac{C}{\Delta\dot{n}} = 10^{11.49\pm0.14}\mathrm{B}\cdot\mathrm{d/mL} \quad (3)$$

where B·d/mL stands for Bacteria times day per milliliter.

This result can be used as a basis for future constructions of MAI and it serves to determine the number of bacteria in samples from MAI data. Apart from that application the result can be given an intuitive interpretation:

The inverse of $10^{11.49}$, which is 3×10^{-12}, would be the refractive index change caused by just one bacterium in 1 mL during 1 day. It may be useful to get an intuitive idea of the order of magnitude of the corresponding chemical throughput that causes this tiny change of refractive index: one does not know how this index change is distributed among the consumption of different chemical species in the culture medium. But if one assumes, just for simplicity, that the main contribution comes from consumption of glucose one may conclude, using $\partial n/\partial c_{\mathrm{glucose}} = (32 \pm 6)$ mL/mol (see Jardim et al. 2003), that one bacterium of BCG consumes $10^{-13.0\pm0.2}$mol per day. That is approximately 7×10^5 molecules per second. In terms of mass (considering again only glucose) the bacterium consumes approximately 3×10^1 times its own mass per day (assuming the geometry of BCG to be a cylinder of 0.5 μm diameter and 3 μm height and a density of approximately lg/mL).

The exponential fitting of BCG data curves permits a determination of the time constant of bacterial growth. The average time constant for BCG in 7H9-OADC determined from 20 samples was found to be

$$\bar{\tau}_{\mathrm{BCG:7H9}} = (2.2 \pm 0.4)\mathrm{d} \quad (4)$$

That means the bacterial population duplicates every 1.5 days. So during its duplication time the bacterium consumes approximately 5×10^1 times its own mass.

The experiments with *M. smegmatis* have much fewer data points and the uncertainties are large. But the results are compatible with what one expects from a quicker species:

$$\left(\frac{C}{\dot{n}}\right)_{\mathrm{M.Smegmatis}} = 10^{10\pm2}\mathrm{B}\cdot\mathrm{d/mL} \quad (5)$$

$$\bar{\tau}_{\mathrm{M.Smegmatis}} = (0.3 \pm 0.2)\mathrm{d} \quad (6)$$

M. smegmatis was also measured in Modified Mueller Hinton culture medium and a similar behaviour was found. In a preliminary test it was also verified that the MAI works perfectly well if one adds antibiotics to the culture medium in minimal inhibitory concentration.

The above results can be used to evaluate the number of bacteria necessary to get a visible signal after a stipulated time. The slowly growing species BCG may serve as a model for *M. tuberculosis*: The minimal time derivative $\Delta\dot{n}$ that can be distinguished from the error with the present apparatus is approximately $6 \times 10^{-7}\mathrm{d}^{-1}$. This corresponds to $1.8 \times 10^5 (=6 \times 10^{-7} \times 10^{11.47})$ active mycobacteria in the sample at detection time. If one wants a detection time of 8 days one needs $4.7 \times 10^3 (=1.8 \times 10^5 \times \mathrm{e}^{-8/2.2}$ bacteria at the beginning of the experiment. It would be desirable to reduce this number to something like 1×10^2 There are essentially two ways of getting to smaller detectible numbers of bacteria: one may change the geometry of the sample holder in such a way that less culture medium is used so that a given number of bacteria corresponds to a higher concentration, and one may try to reduce the errors of phase detection and sample preparation so that the smallest detectable $\Delta\dot{n}$ is lowered. It should be possible to gain factors of 6 or 8 with both strategies, so that the overall gain may reach 5×10.

Conclusion

It was shown that mycobacterial growth of slowly growing species can be observed interferometrically in several samples simultaneously. For *M. bovis* BCG in 7H9-OADC it was shown that bacterial concentration and rate of refractive index change are proportional. The constant of proportionality as well as the time constant of bacterial growth were determined for *M. bovis* BCG in 7H9 culture medium and *M. smegmatis*. These values may be used to determine the number of bacteria in samples from experimental values of index changes. They are also basic parameters for future constructions of MAI of the present apparatus is high enough to use this prototype for practical research work and for tests of bacterial susceptibility but enhancement of sensibility is still desirable.

Acknowledgments We thank the Serviço Brasileiro de Apoio às Micro e Pequenas Empresas (SEBRAE), the Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG), and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support, which has been fundamental for the construction of the MAI. We also thank Marlei Gomes da Silva for all the technical aid in mycobacteriology and Luiz do Carmo da Silva for measurements with the flow cytometer.

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