A rapid unravelling of mycobacterial activity and of their susceptibility to antibiotics

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The development of antibiotic-resistant bacteria is a worldwide health-related emergency that calls for new tools to study the bacterial metabolism and to obtain fast diagnoses. Indeed, the conventional analysis timescale is too long and affects our ability to fight infections. Slowly growing bacteria represent a bigger challenge, since their analysis may require up to months. Among these bacteria, *Mycobacterium tuberculosis*, the causative agent of tuberculosis has caused, only in 2016 more than 10 million new cases and 1.7 million deaths. We employed a particularly powerful nanomechanical oscillator, the nanomotion sensor, to characterize rapidly and in real time a tuberculous and a non-tuberculous bacterial species, *Bacillus Calmette-Guérin* and *Mycobacterium abscessus* exposed to different antibiotics.

Here, we show how high speed and high sensitivity detectors, the nanomotion sensors, can provide a rapid and reliable analysis of different mycobacterial species, obtaining qualitative and quantitative information on their response to different drugs. This is the first application of the technique to tackle the urgent medical issue of mycobacterial infections, evaluating the dynamic response of bacteria to different antimicrobial families and the role of the replication rate in the resulting nanomotion pattern. In addition to a fast analysis, which could massively benefit patients and the overall healthcare system, we investigated the real-time response of the bacteria to extract unique information on the bacterial mechanisms triggered in response to the antibacterial pressure, with consequences both at the clinical and at the microbiological level.
Introduction

Providing fast and reliable antibiotic susceptibility data, which allows initiating prompt and appropriate therapy schedules, is a main task of the clinical laboratory. While molecular techniques (i.e. MALDI-TOF Mass Spectrometry (MS) or PCR-based gene amplification)(1, 2) have radically changed the time-frame for Gram positive (+) and negative (-) bacterial identification (ID), most of current methods for antimicrobial susceptibility testing (AST) are still based on fluorescence staining or phenotypic assays(1, 3) which may require days, or even weeks, depending on the bacterial species. In conventional clinical workflow, the sample (e.g. blood, spinal fluid, urine, feces, nasal or throat swabs) is harvested from patients, streaked on agar nutrient media and, after 12-24 hours incubation, transferred for further analysis. This unavoidable culture step results in isolation of microorganisms in liquid cultures containing up to $10^8$ – $10^9$ Colony Forming Units (CFU)/ml.(4) ID and culture-based AST are performed after this incubation time, and can last more than 24 hours.(5) Similar protocols are commonly used for slowly growing bacteria such as mycobacteria but, in these cases, the incubation time can be longer than a week (e.g. in the MGIT 960 system), and more than one month may be required to obtain ID and AST.(5) This long timeframe, under the pressure of life-threatening infections, often results in imprudent use or misuse of antibiotics. Early appropriate therapy will significantly reduce the spread of pathogenic bacteria into the population. This will lead to higher patient survival rate, lower distress and an optimized use of the limited resources of healthcare systems.(6, 7)

Many options are emerging to achieve a rapid, accurate and cost-effective pathogen characterization of bacterial response to drugs, ranging from molecular to rapid phenotypic techniques to plasmonic single cell assays.(8-11) The conventional AST molecular techniques rely mainly on the determination of the genetic fingerprint associated with resistance to a specific antibiotic, including real-time PCR (RT PCR), DNA microarrays, Next-generation sequencing (NGS), cell lysis-based approaches, whole-genome sequencing, and MALDI-TOF MS.(12-16) Even though the
aforementioned techniques can provide fast and high-throughput results, they still present profound drawbacks. For instance, their outcome is strongly dependent on specific drug-target genes, which can only be indicative of the actual antibiotic resistance, leaving a large gap for drug phenotypic response.

Novel single-cell techniques are have gained importance in the last decade in particular for cancer-related applications. Nevertheless, the analysis of bacterial susceptibility requires the concurrent evaluation of hundreds or thousands of bacteria to assess response at the population level. Thus, their application is mostly limited to the research laboratory and their low throughput hinders their transition to a clinical susceptibility test.

Most commercial phenotypic assays rely on bacterial replication to deliver a correct AST and the timescale for antibiogram determination may range from days, for rapidly replicating bacteria, to weeks or months, for slowly growing and fastidious germs. To shorten turnaround times and costs, several automated systems are now available, including, for instance, MicroScan WalkAway (Beckman Coulter), BD Phoenix (Dickinson Becton), Vitek-2 (bioMérieux), SIDECAR (Alifax). The outcome is a relatively rapid susceptible/resistant response, which is available to the clinician as soon as the second day from the first examination of the sample, with large advantages for treatment, but still too long for severe infections (e.g. meningitis, sepsis). (1, 3) In addition, most of these techniques provide no clue on the particular drug effect or on the counter mechanisms employed by bacteria to react to antibiotic compounds.

Among the alternatives to conventional microbiological assays, small and extremely sensitive nanomechanical oscillators stand out as very promising candidates. At first, such devices were employed in Atomic Force Microscopes (AFM) to study dynamic behaviour in cells or proteins. Nowadays they are increasingly used for the detection of very small masses or for nano-stress sensing in molecular biology and their sensitivity and versatility...
is exploited in lab-on-a-chip devices to measure bio-molecular interactions (27) or mass variations in biological systems, including bacteria. (28, 29) Two major factors limit most of the available nanomechanical systems: (i) they require air or vacuum conditions for the measurements; (ii) they need bacteria to replicate directly on the sensors, to determine cell viability through mass change or local stress alterations. This reflects on the time required to perform analysis, and on the range of information that can be obtained, based on life or death assessments.

Very recently, we have introduced a new way of exploiting the capabilities of cantilever nanosensors: the nanomotion sensor (NMS) (30, 31). By exploiting the intimate link between life and motion, measuring the fluctuations of flexible cantilevers that act as solid support for microorganisms, we can monitor in real time the metabolism of living organisms. (32) The sensor’s fluctuations strongly depend on the microorganisms’ metabolic activities, which combine energy consumption, cell vibration and movement. The sensitivity of the technique allows detecting the energy consumption of few ATP molecules, as demonstrated in previous works using Finite Elements Modelling or studying conformational changes in quaternary protein structures. (31, 33) Thus, the NMS can been used to evaluate the fluctuations of a very limited number of viable specimens (single mammalian cells or tens of bacteria). (32, 34-37)

By measuring the fluctuations as a function of time and of external conditions, the NMS delivers a unique insight on microorganisms response to environmental, chemical or physical stimuli. (32) The amplitude and the low frequency of these fluctuations (<1 kHz) help circumventing the major limitations of current nanomechanical sensors: the NMS can be used under various conditions (i.e. buffer solutions or growing media), and the viable specimens are attached on both sides of the sensor, reducing complexity and cost of each experiment. In addition, due to the time resolution of the NMS, these experiments allow investigating in real-time and with sensitivity in the Angstrom-to-micron range (33) the evolving metabolism of the adsorbed bacteria, long before a single replication (30, 31), suggesting that the analysis timescales are only marginally dependent on
bacterial duplication rates, as demonstrated by studying *B. pertussis*, which replicates in 48 hours

(38). In addition, the sensitivity of these sensors allows extracting a measurable signal from groups of less than 100 bacterial cells, leading to a drastic reduction of incubation times. Overall, the outcome is a quantitative evaluation of the real bacterial response to the applied stimuli, on a time-scale (hours) comparable to the fastest molecular analyses.

(39) Furthermore, the real-time monitoring of a nanomotion susceptibility test (N-AST) reveals a different kind of information on the bacterial behaviour compared to conventional phenotypic and genetic studies, with the potential to improve therapeutic interventions, which are paramount for clinical decisions and, hopefully, therapeutic outcomes, as demonstrated in previous works on rapidly growing bacteria.

(40) For this reason, coupling the NMS with other conventional techniques could help to better unravel which molecular processes arise to account for specific observed motion-change behaviour.

Results

We exploited the capabilities of the NMS to study slowly growing *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) and the fast growing nontuberculous mycobacterium (NTM) *Mycobacterium abscessus*.

(41) In particular, the former species belongs to the *M. tuberculosis* complex (MTC), which includes *M. tuberculosis* (Mtbc), *M. africanum*, representing a growing medical emergency in both developing and developed regions.

(42) By studying their nanomotion, we characterized the interaction between these two species and three antibacterial drugs, and were able to determine in a matter of hours their susceptibility, calculating their minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC). Furthermore, we exploited the real-time analysis of the NMS to evaluate the peculiar responses of these bacteria to the different antibacterial agents. We chose to work with a relatively large number of bacteria on the sensor (approximately between 100 and 1000 cells) to ensure a good population-level analysis of...
the susceptibility and to average out the possible presence of outliers or single naturally resistant microorganisms.

In preliminary experiments, we determined whether NMS preparation protocols influenced the viability of BCG and *M. abscessus* in the MGIT medium. Overall, we kept the bacteria for at least 200 min in the analysis chamber by monitoring the oscillations over time. As shown in Figure 1, the variance of the nanomotion signals remained constant over time, indicating that the bacteria were viable for the entire control experiment.

**BCG**

We performed a series of experiments involving exposure of BCG to RIF or INH, two first line anti-TB drugs inhibiting the DNA-dependent RNA polymerase, and specific enzymes implicated in cell wall synthesis, respectively.\(^{(42, 43)}\) We selected this species because it belongs to the MTC, is not dangerous for humans, and can be safely handled in a Biosafety Level 2 laboratory, constituting a safe NMS testing ground for the study of more dangerous mycobacteria, including Mtb.

When exposed to bactericidal doses of these agents, the BCG produced a sharp reduction of the sensor’s fluctuations, underlining the drug’s activity. In a typical experiment, the outcome of the exposure to a bactericidal dose of INH (2 \(\mu\)g/ml) could be determined in less than 30 min (Figure 2). On the other hand, after exposure to a high dose of RIF (0.7 \(\mu\)g/ml in Figure 3), we observed a reduction in the sensor’s movement, demonstrating a slower bactericidal effect and confirming bacterial death which required almost 2 hours.

The subsequent step in the analysis of the response of BCG to these antibacterial agents consisted in a series of dose-dependence experiments. The results, summarized in Figure 4a for INH and 4b for RIF, indicate for each experiment the relative reduction of the nanomotion fluctuations. In both cases, the linear fit of the sigmoid curve was used to determine the MIC and MBC values. The
obtained values were MIC 0.09+0.03 μg/ml and MBC 0.17+0.03 μg/ml for INH; MIC 0.15+0.07
μg/ml and MBC 0.4+0.07 μg/ml for RIF. The MICs of BCG Pasteur determined by NMS differed
by about 1-fold dilution from those determined by conventional proportion methods (0.2 versus
0.09 μg/ml for INH, and 0.063 versus 0.15 μg/ml for RIF, respectively).(44-46) Such discrepancy
between the conventionally measured MIC and the N-AST is something that has been also
highlighted in previous experiments involving rapidly growing bacteria.(31, 39) This difference can
be interpreted by invoking many factors, such as growth conditions, measurement geometry or
temperature. It must be noted that conventional and NMS assays monitor different metabolic
parameters. In most conventional analyses, the MIC is identified by the bacterial ability to replicate,
while for the NMS, this concentration is associated to the reduction of the sensor’s fluctuations
associated to alterations in the bacterial membrane elasticity(36, 37) or to their internal metabolic
activity. Indeed, while the information content is the similar, the concentration at which one or the
other phenomenon occurs can be different.

In addition to these quantitative susceptibility results, performing a real-time analysis on antibiotics
susceptibility allowed us to evaluate how the drug pressure influenced the investigated
microorganisms, including their peculiar response patterns and typical timescales. For instance,
INH exposure, even if using sub-MIC concentrations, caused an immediate response of BCG,
which was registered as a fluctuation intensity increase that lasted 10-15 min before a rapid decay
of the movements. After few tens of minutes, if the concentration was not bactericidal (i.e. 0.025
μg/ml in Figure 5a), the variance of the fluctuations recovered their intensity and, returned to values
comparable to those measured before the antibiotic injection. This entire response pattern did not
last more than 20 minutes. On the other hand, if the drug concentration was higher than the MBC
(e.g. 1 μg/ml, Figure 6), the response was more complex. After an initial rise of the oscillations, the
movements rapidly decreased to lower values for up to 25 min, followed by few seconds of wide
fluctuations. This biphasic pattern repeated itself several times for more than 1 h, until the
fluctuations stabilized to low values, indicating the death of the BCG. A possible interpretation of this pattern is related to BCG clumping: these bacteria exploit their waxy coating to form cell aggregates not completely dissolved during sample preparation procedures. In such clumps, external bacteria are expected to be metabolically more active than internal ones, partially shielding them from some environmental attacks. In this view, the bactericidal antibiotics could kill, at first, the cells of the external layer, and then the internal bacteria would be activated, resulting in the movement-stasis pattern we observed and measured. Clumping is an already known defense mechanism in microbiology and can be found in many different species, such as Candida albicans, or in self-aggregation in Escherichia coli or in flocculation in Saccharomyces cerevisiae (47-50) but it has never been reported in this way for BCG.

While we identified these peculiar movements in INH-exposed BCG, these were never seen in RIF experiments (Figure 5b), suggesting that this behavior is strictly dependent on the response mechanism of BCG to INH. Indeed, the response against 0.07 μg/ml of RIF did not involve a strong initial reaction to the drug, with fluctuations lasting from 1 to 2 h. In some cases, after minutes from drug exposure, we measured a temporary reduction of movements, which lasted up to 30 min, followed by full recovery of the nanomotion fluctuations. Furthermore, in RIF-exposed BCG, we never identified the oscillating patterns seen for INH. A possible interpretation of the different reported behavior respect to the two different drugs could rely on their different time-scale of the effect. While INH affects the cell wall synthesis, its effect is quite fast and the shielding of the clumping could produce a visible transitional oscillation. On the other hand, since RIF targets RNA polymerase consequently blocking protein translation, its longer timescale could cover the aforementioned effect.

M. abscessus
To investigate the response of drugs against *M. abscessus*, we used the protein synthesis inhibitor, amikacin (AK), because this organism is known to be resistant to INH and RIF. In the presence of a bactericidal concentration of the drug (10 μg/ml in Figure 7a), *M. abscessus* did not show an immediate response, without alterations of the fluctuations for the first 30 min, followed by a sharp decrease of the movements between 50 and 70 minutes, after which the fluctuations did not recover. When non-bactericidal doses were used (1 μg/ml in Figure 7b), a lag phase of approximately 10-25 min was observed, followed by a full recovery of the nanomotion signal. This trend is in line with the NMS response of *Staphylococcus aureus* exposed to cefoxitin(31) or *E. coli* to ceftriaxone(39). It is worth noting that the response time of *M. abscessus* to AK (50-70 minutes) is longer than in the BCG-INH case (20-30 min) and comparable with the BCG-RIF timescales (80-100 min). This can be attributed to the different mechanisms of action of AK compared to INH. Indeed, INH inhibits the cell wall synthesis and is expected to have a more rapid effect than RIF and AK, which inhibit the protein synthesis. Remarkably, the response times of the BCG and *M. abscessus* are similar to the typical timescales of the experiments involving rapidly replicating bacteria (between 15 and 60 minutes for both *E. coli* and *S. aureus*(31, 39)). This clearly demonstrates the nanomotion sensor’s independence to the replication rate of the specimens under investigation. Furthermore, it also depicts the range of interesting new information that the technique can provide.

As done for BCG, we performed a series of dose-dependence measurements, in order to obtain quantitative susceptibility results for *M. abscessus* exposed to AK (Figure 4c). The linear fit for the sigmoid curve indicated a MIC of 1.7+0.6 μg/ml and a MBC of 7.8+0.6 μg/ml. These concentrations are in good agreement (within one dilution) with those present in literature for this reference *M. abscessus* strain (3.1 versus 1.7 μg/ml).(51, 52)
M. tuberculosis and NTM are the causative agents of extremely dangerous infections, such as TB, which caused in 2016 more than 10.4 million new cases and 1.7 million deaths in developing and industrialized countries (WHO Global TB Report 2017). A fast diagnosis of these bacteria will allow a more specific, tailored treatment, more effective, better tolerated by the patient, and less likely to produce relapses. An early diagnosis of infections from slowly growing bacteria could be lifesaving and could significantly reduce the spread of harmful pathogens into the population, leading to higher survival rate, lower distress and an improved use of the limited resources of the healthcare system. (53, 54)

This work shows how the NMS can be used to obtain a rapid and reliable investigation of the MTC and NTM, with possible impact in the early clinical and diagnostics fields. Indeed, while the clinical application of the NMS has already been demonstrated on E. coli, (39) its importance to the study of slowly growing germs can be safely suggested. In addition, the speed of the nanomotion analyses can allow a rapid screening of innovative molecules and antitubercular agents, to accelerate and reduce the costs for drug discovery and drug development. (55, 56) Furthermore, the NMS can highlight the specific behavior of slowly growing bacteria during antibiotic treatment, evidencing cooperative vibrations and activity reduction, which could not be determined using other phenotypic techniques. This provides a better overview of the metabolism of slowly growing bacteria in models more closely mimicking in vivo microenvironments and of their dynamic responses to external stimuli, which is of great importance in medical and pharmacological TB research. These results illustrate how N-AST can become a reliable and rapid investigation tool for slowly growing bacterial species, providing also a new insight into the behavior of these bacteria during antibiotic treatment.

The possibility to combine NMS and molecular biology assays would deliver a thorough comprehension of the bacterial resistance mechanisms, which could provide invaluable information.
to produce newer, more targeted drugs and anti-mycobacterial agents, to fight some of the deadliest
diseases of our times.

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Materials and methods

262 Substrates, enzymes and reagents

263 Chemicals, phosphate buffered saline (PBS), glutaraldehyde and antibiotics rifampicin (RIF),
isoniazid (INH) and amikacin (AK), all with analytical grade, were obtained from Sigma-Aldrich
(St Louis, MO). MGIT 960 (MGIT) tubes (Becton-Dickinson Microbiology Systems, Sparks, MD)
were used to obtain the bacterial culture medium.

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Bacterial preparation and isolation

269 *Mycobacterium bovis* BCG [American Type Culture Collection (ATCC) 27291 (Pasteur strain)]
cells, frozen at -70°C, were thawed, and grown in Löwenstein Jensen slants at 37°C for 3 weeks.

270 Few colonies were transferred in tubes containing 500 μl of MGIT medium. Since the BCG tends to
form clumps that are difficult to disaggregate, before transferring the bacteria-rich medium to the
nanomotion experiments we vortexed the tubes to ensure that any large bacterial clump was
dissolved or reduced. Still, as shown in Figure 8, small BCG clumps were present on the sensors.

274 The MIC of BCG Pasteur was determined using the proportion method. The characterizations
showed that, in good agreement with the values present in literature, the MICs were 0.2 μg/ml for
INH(45) and 0.063 μg/ml for RIF(46), respectively, with an uncertainty of one dilution.

278 *Mycobacterium abscessus* [ATCC 19977] cells frozen at -70°C were thawed, and the specimens
were grown in MGIT medium at 37°C for 3 days under continuous agitation. 1 ml of the bacteria-
rich medium was centrifuged (5000 rpm for 5 min), the pellet was re-suspended in PBS and transferred directly to nanomotion experiments. The susceptibility values present in literature for ATCC 19977 towards amikacin detail a MIC of 3.1 μg/ml, with an uncertainty of one dilution. (52)

**Nanomotion experiments**

The nanoscale movements of the microorganisms induced dynamic deflections of the NMS, which we monitored in real-time, using the laser-based signal transduction typically used in most AFMs. We collected these fluctuations in a time-dependent chart of the vertical movements of the sensor, which appeared as a coloured noise signal, superposition of a large number of vibrations. Overall, a typical experiment lasted more than 2 hours divided in data chunks of at least 30 minutes. The control experiments were carried out for at least 4 hours. In each experiment, the sensors were followed in real-time using optical cameras.

We used a commercial Nanowizard III microscope (JPK Instruments, Berlin, DE) and custom homemade devices developed in the Laboratories of Living Matter Physics at the EPFL (LPMV-EPFL). The sensors chosen for the JPK experiments were the ONP-10 tipless AFM cantilevers from Bruker (Bruker Nano Inc., MA, USA). For the homemade devices, we employed the SD-qp-CONT tipless cantilevers (Nano and More Gmbh, DE). For all experiments, we used sensors with spring constant in the range of 0.08±0.03 N/m, which demonstrated a good sensitivity coupled with geometrical properties that ensured space for an adequate number of bacteria (as controlled through optical images throughout each experiment). The data from the Nanowizard III microscope were collected using the JPK software using a 10 kHz acquisition rate, while the data from the experiments on the custom devices were collected using a USB-4431 DAQ card (National Instruments, USA), using a 20 kHz acquisition rate. Remarkably, as a demonstration of the solidity...
of the analysis technique, we were able to compare the results obtained with the two different instrumentations, which exhibited similar sensitivity and noise levels.

To enhance bacterial adhesion, as established and detailed in many previous works, we chemically treated the sensors. In literature, there are examples of fibronectin, poly-lysine, polyethylene imine, APTES, glutaraldehyde, concanavalin or even collagen used for this purpose. In comparison with the specimens analyzed in some previous works, the thick, hydrophobic and waxy cell walls of mycobacteria required new immobilization strategies and the use of specific culture media and drugs. In the present work, we exposed the sensors to 0.5% glutaraldehyde for 15 min, followed by rinsing in ultrapure water and air-drying. This immobilization protocol allowed mycobacteria to adhere on the sensors with little influence on their metabolic activity (as demonstrated by the control experiments depicted in Figure 1).

We equipped both instruments with closed custom analysis chambers, made of plastic or polydimethylsiloxane (PDMS), which allowed performing experiments in liquid while changing the medium with very low noise. For the subsequent immobilization of the living bacteria on the sensors, we strictly followed a protocol that ensured a remarkable adhesion of the cells on the sensors. Since the growth media often contain complex molecules that could passivate the chemical functionalization on the sensor, we washed twice in PBS an aliquot of MGIT-containing bacteria (by centrifuging cells at 3000xg for 5 min, and re-suspending the pellet in 100 µl of PBS with a final OD that ranged from $10^6$ to $10^8$ CFU). In order to let the bacteria adhere on the sensor we deposited a droplet (typically 20 µl) of the bacteria-rich PBS on the sensor and incubated at room temperature for about 15 min. This protocol allows the formation of a roughly uniform distribution of bacteria on the sensor surface, with an innate variability in their number and spatial distribution. Thereafter, we mounted the sensor on the holder and inserted it into the analysis chamber, carrying 2 ml of MGIT medium for
As shown in the optical images of the sensors during the experiments (Figure 8), an approximate estimate of 100 to 1000 cells adhered to the NMS, while more complex procedures must be carried out to obtain a quantitative determination. (31) It is worth highlighting that the immobilization protocol produces a variability in the absolute value of the oscillations which are different for each experiment, but the strong attachment ensures that the number and position of the bacteria is well-preserved throughout each single analysis.

As detailed in previous works, to generate dose-dependence curves we compared several parallel experiments in which the bacteria were exposed to different concentrations of antibiotic drugs. To obtain these graphs we focused on the relative reduction of the fluctuations compared to the maximum variance measured before the exposure to the chosen drug concentration. In this way we were able to define a comparative measure of the bacterial response and to compare and average the many experiments we have performed. Each experiment was performed at least in triplicate, lasted at least 2 hours and comprised three steps: i) preparation of viable bacteria in medium (BCG or *M. abscessus* in MGIT medium); ii) exposure to the desired drug concentration (INH, RIF or AK); iii) exposure to a high bactericidal drug concentration. (31) The concentration dependence curve was then fitted with a sigmoid curve interpolation, bearing a similar information content of a conventional antibiogram. By performing a linear fitting of the central part of the sigmoid, we calculated two concentration values which can be associated to the minimum inhibitory concentration (MIC - the drug concentration that inhibits visible bacterial growth) and the minimum bactericidal concentration (MBC - the concentration that ensures the death of 99.9% of the bacteria). (31) New AST assays, intended as substitute of conventional microbiological procedures should provide these two concentrations, and this is one of the main advantages of phenotypic assays compared to faster molecular counterparts. Indeed, just as for the conventional values, at drug concentrations below the NM-MIC, the fluctuations of the cantilever are largely unaffected, while at values higher than the NM-MBC the movements are largely reduced.
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353 **Statistical analysis**

354 The nanomotion data is presented as acquired, with a simple linear fit to remove any long-term drift from the traces. The variance calculations are presented as histograms + SD calculated on the corresponding fluctuations over the corresponding raw data, but the trends are indicative of at least three independent sample preparations.

358 Each datapoint of the dose-dependence graphs was obtained from minimally three independent experiments and the graphs were drawn following the procedures detailed elsewhere. (31) The error bars represent the variability of the different experiments performed at each concentration.
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Data availability

All the presented data is available upon reasonable request. Figures 1, 2, 3, 5, 6 and 7 present raw data.

All data collection and analysis were performed using custom Labview code, which is freely available upon reasonable request.

Author contributions
GL, AM and LV performed the nanomotion experiments; SK and GD provided the nanomotion sensor infrastructure; GL, SK and GD developed and optimized the methodology; GL and LV developed the software; AM and LF provided and characterized the BCG; KB and RAF provided and characterized the *M. abscessus*; GL, LV, SD and MG performed the data analysis; GL, LV, AM, SD and MG wrote the manuscript; all authors read and commented on the manuscript.

**Transparency declaration**

The authors declare no competing interests.
References


Figure captions

Figure 1. Control experiments involving BCG and M. abscessus. Typical data patterns (performed minimally in triplicate) of BCG (panel a) and M. abscessus (panel b) in MGIT medium.

The fluctuations are present for more than 200 minutes.

Figure 2. Nanomotion experiments on BCG exposed to an over-MIC dose of INH. Top panel. Typical 10 minutes segments of the sensor’s fluctuations: before the exposure to INH (left); immediately after the exposure to INH at 2 µg/ml (center); 20 minutes after the exposure to INH, when the movement reduction has stabilized. Lower panel. Histogram of the corresponding variance of the fluctuations. The graph is representative of minimally 5 independent experiments which produced similar results. The error bars in the variance histogram represent +- SD of the corresponding fluctuations over the shown 10-minute time-period.

Figure 3. Nanomotion experiments on BCG exposed to an over-MIC dose of RIF. Top panel. Typical 20 minutes segments of the sensor’s fluctuations: before the exposure to RIF (left); immediately after the exposure to RIF at 0.7 µg/ml (center); 80 minutes after the exposure to RIF, when the movement reduction has stabilized. Lower panel. Histogram of the corresponding variance of the fluctuations. The graph is representative of 3 independent experiments which produced similar results. The error bars in the variance histogram represent +- SD of the corresponding fluctuations over the shown 20-minute time-period.
**Figure 4. Dose dependence experiments.** Panel a. Normalized variance calculated from the deflections collected by exposing the BCG to different INH concentrations. Panel b. Normalized variance calculated from the deflections collected by exposing the BCG to different RIF concentrations. Panel c. Normalized variance calculated from the deflections collected by exposing the *M. abscessus* to different AK concentrations. The concentration values can be well fitted with a sigmoid function, which is comparable with the antibiogram plots, obtained using conventional microbiological techniques. The MIC and the MBC of towards the bacterial species can be obtained by calculating the tangent of the sigmoid fit at half height (black dashed line). Each data-point represents the average of a minimum of 3 independent experiments performed using the same drug concentration. The error bars represent the variability of the different experiments performed at the same concentration. In each graph, the experiments involving sub-MIC drug concentrations are represented as a single data-point, which summarizes all these experiments.

**Figure 5. Nanomotion experiments on BCG exposed to a sub-MIC dose of INH and RIF.** Panel a: Top panel. Typical 20 minutes segments of the sensor’s fluctuations: before the exposure to INH (left); immediately after the exposure to INH at 0.025 μg/ml (center); 140 minutes after the exposure to INH, when the movement has stabilized. Lower panel. Histogram of the corresponding variance of the fluctuations. Panel b: Top panel. Typical 20 minutes segments of the sensor’s fluctuations: before the exposure to RIF (left); immediately after the exposure to RIF at 0.07 μg/ml (centre); 95 minutes after the exposure to RIF, when the movement has stabilized. Lower panel. Histogram of the corresponding variance of the fluctuations. Each graph is representative of minimally 3 independent experiments which produced similar results. The error bars in the variance histogram represent +- SD of the corresponding fluctuations over the shown 20-minute time-period.
Figure 6. Time-resolved analysis of the BCG response to INH. Typical data pattern of the response of BCG to a bactericidal dose of INH (1 μg/ml). Over a 140-minute period, the fluctuations increase and decrease in amplitude, highlighting the bacterial response to the antibiotic pressure. The graph is representative of 3 independent experiments in which this feature was evidenced.

Figure 7. Nanomotion experiments on M. abscessus exposed to AK. Panel a: Top panel. Typical 20 minutes segments of the sensor’s fluctuations exposed to an over-MIC dose of AK: before the exposure to AK (left); immediately after the exposure to AK at 10 μg/ml (center); 50 minutes after the exposure to AK, when the movement has stabilized. Lower panel. Histogram of the corresponding variance of the fluctuations. Panel b: Top panel. Typical 20 minutes segments of the sensor’s fluctuations exposed to a sub-MIC dose of AK: before the exposure to AK (left); 30 minutes after the exposure to AK at 1 μg/ml (center); 90 minutes after the exposure to AK, when the movement has recovered and stabilized. Lower panel. Histogram of the corresponding variance of the fluctuations. Each graph is representative of at least 3 independent experiments which produced similar results. The error bars in the variance histogram represent +- SD of the corresponding fluctuations over the shown 20-minute time-period.
Figure 8. Optical images of bacteria-bearing sensors. Typical optical images of rectangular (left panels) and triangular sensors (right panels) bearing BCG (panel a) and *M. abscessus* (panel b). The optical images show the BCG clumping. The scale bars indicate 50 μm.