A Single-Cell Perspective on Non-Growing but Metabolically Active (NGMA) Bacteria

Giulia Manina and John D. McKinney

Abstract A long-standing and fundamental problem in microbiology is the non-trivial discrimination between live and dead cells. The existence of physically intact and possibly viable bacterial cells that fail to replicate during a more or less protracted period of observation, despite environmental conditions that are ostensibly propitious for growth, has been extensively documented in many different organisms. In clinical settings, non-culturable cells may contribute to non-apparent infections capable of reactivating after months or years of clinical latency, a phenomenon that has been well documented in the specific case of *Mycobacterium tuberculosis*. The prevalence of these silent but potentially problematic bacterial reservoirs has been highlighted by classical approaches such as limiting culture dilution till extinction of growing cells, followed by resuscitation of apparently "viable but non-culturable" (VBNC) subpopulations. Although these assays are useful to demonstrate the presence of VBNC cells in a population, they are effectively retrospective and are not well suited to the analysis of non-replicating cells per se. Here, we argue that research on a closely related problem, which we shall refer to as the "non-growing but metabolically active" state, is poised to advance rapidly thanks to the recent development of novel technologies and methods for real-time single-cell analysis. In particular, the combination of fluorescent reporter dyes and strains, microfluidic and microelectromechanical systems, and time-lapse fluorescence microscopy offers tremendous and largely untapped potential for future exploration of the physiology of non-replicating cells.

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1 Introduction

The second half of the nineteenth century marked the beginning of a new era in microbiology. A number of techniques were introduced to select and maintain pure bacterial cultures, resulting in the emergence of the "germ theory" of disease causation followed by the establishment of a system of proofs to link a specific microbe to a specific diseases state known as "Koch's postulates." It was during this period that microbes like *Mycobacterium tuberculosis* and *Vibrio cholerae* were identified as the etiological agents of specific communicable diseases. At the same time, the introduction of differential-staining techniques dramatically improved the resolution of features that could be achieved by optical microscopy, allowing the user to distinguish the physical and morphological characteristics of cells in order to assess the viability and death of individual bacteria. In subsequent years, fluorescent dyes—for example, dyes that specifically stain nucleic acids, were broadly used in conjunction with epifluorescence microscopy or flow cytometry to assess total bacterial counts or specific metabolic activities, such as respiration or intracellular enzyme activities (Roszak and Colwell 1987). More recently, microbiologists have relied on commercial kits based on combinations of fluorescent reagents to differentiate between live and dead cells. Despite the widespread application of such "live/dead" reagents, it is important to note that these reagents provide only an indirect assessment of cellular viability, as they typically measure cellular physiological parameters (e.g., specific enzyme activities, cell envelope permeability, etc.) that are assumed but not necessarily proven to correlate with live/dead cell states.

The application of fluorescent reagents and single-cell measurements, often involving optical microscopy or flow cytometry, led to the realization that bacterial populations are phenotypically heterogeneous. This realization raised difficult questions about the relationship among the metabolic state, growth rate, and viability of individual cells. Many techniques for counting the number of live cells in a bacterial population, such as the standard method for plate counts of

colony-forming units (CFU), rely on bacterial growth for detection. Although growth-dependent techniques unequivocally detect viable cells and exclude dead cells (which cannot grow, by definition), they may detect only the most active subpopulations of cells within a culture and may underestimate the actual number of living cells, because some viable cells may fail to grow during the time window of observation. Furthermore, these techniques typically provide only a retrospective view of the physiology of cells within a population—for example, by counting colonies of *M. tuberculosis* that do not become visible until several weeks after plating. These issues became increasingly problematic when investigators realized that bacterial populations often contain large numbers of cells that appear to be viable, inasmuch as they are physically intact and may even display metabolic activity, yet which fail to grow and divide during the period of observation. Despite decades of intensive research, the significance of these so-called "viable but non-culturable" (VBNC) cells remains elusive (Mukamolova et al. 2003; Oliver 2010; Trevors 2011), and in only a few cases has it been shown unequivocally and reproducibly that VBNC cells can be converted to actively growing cells by the application of a specific stimulus.

When bacteria do not grow under conditions that are apparently favorable for growth, a number of questions arise. Is a non-growing cell truly dead, in the sense that it has lost all capacity to resume growth in future? What is the metabolic state of a cell that remains intact but fails to grow? Can we consider a cell to be "alive" in some meaningful sense if it is metabolically active but non-replicating? For how long can such a non-replicating cell remain capable of resuming growth, and what stimuli might be effective in triggering the resumption of growth? Finally, related to that last question, for how long should we monitor a cell before concluding that it has entered a non-replicating but possibly still-viable state? These questions are not merely of academic interest, as VBNC cells have been implicated in the ability of bacteria to persist in diverse environments, including animal and human hosts.

In general, we can consider a bacterial cell to be "viable" if its genetic information is intact and if it carries out essential metabolic processes, generates ATP, and maintains a metabolic potential required to sustain chromosome replication, RNA transcription, protein translation, balanced increase in biomass, surface synthesis, partition of cytoplasmic macromolecules, and finally division into two daughter cells (Fig. 1). Thus, assessing the dynamics of the molecular events that are necessary to sustain cell survival and proliferation can serve as reliable evidence of cell viability. However, the time scales on which these processes unwind may differ quite substantially between individual cells of the same species. According to this perspective, in order to reach a more thorough and accurate understanding of bacterial physiology, bacteria should be studied not only at the population level but also at the level of individual cells. As most biological assays are based on population-averaged measurements, the latter goal necessitates the development and application of new investigative tools for real-time single-cell analysis of cell physiology and metabolism.

In the following chapter, we will address issues relating to the definition of life and death of individual bacteria, focusing on the still-enigmatic state of

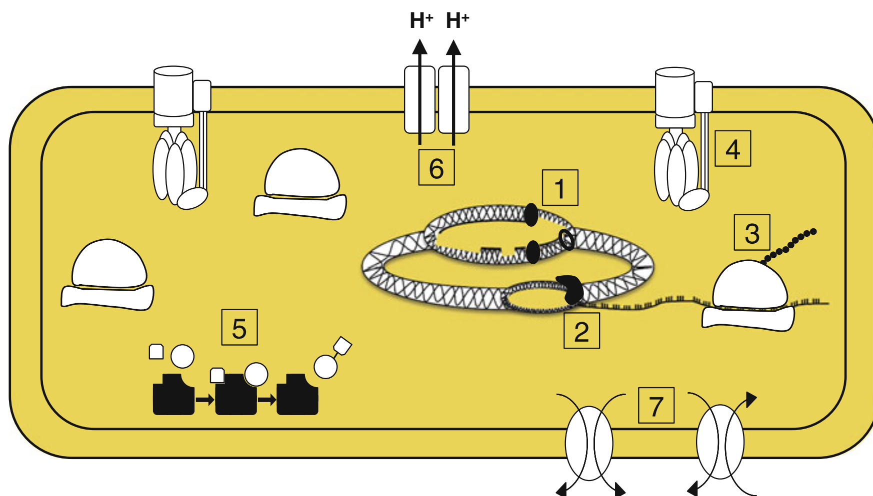

Fig. 1 Schematic illustration of fundamental cellular processes. Cells carry out a number of processes necessary to their survival, including: DNA replication (*1*), transcription (*2*), translation (*3*), ATP synthesis (*4*), enzymatic reactions involved in metabolic processes (*5*), respiration (*6*), symport and antiport of various molecules (*7*). The use of fluorescent reporter-tagging methods to visualize one or more of these intracellular processes, in combination with microfluidic culture systems and time-lapse fluorescence microscopy, can provide direct evidence of ongoing metabolic activity occurring in replicating cells as well as non-replicating NGMA cells

non-culturability of intact and potentially viable cells. We will describe some documented examples of VBNC organisms, and attempt to relate this literature to *M. tuberculosis*, a naturally slow-growing species that may also be capable of long-term survival in a VBNC-like state. We will describe recent progress in the field of mycobacteriology with the aim of understanding the physiology of the dormant and persistent states, both in vitro and in vivo, and we will highlight some classic experiments as well as new technologies. Lastly, we will discuss the use of fluorescent markers for direct, real-time measurements of physiological processes inside individual living cells by time-lapse fluorescence microscopy, and the specific utility of these techniques for the study of non-replicating bacteria.

2 Non-Growing but Metabolically Active (NGMA) Bacteria

2.1 Viable, Dead, or Just "Non-Culturable"? Not a Trivial Question…

The visible processes of bacterial growth and division giving rise to two daughter cells not only ensures the perpetuation of the genetic material but also provides

compelling evidence that the bacterium is alive. On the other hand, demonstrating unequivocally that a bacterium is *not* alive is less straightforward. Ascertaining the metabolic and growth states of individual cells within a microbial community is a highly relevant albeit complex and challenging problem in microbiology that has important implications for microbial ecology and human health. Bacterial populations, even those that are isogenic, are characterized by substantial phenotypic heterogeneity (Balaban 2011; Davidson and Surette 2008; Dhar and McKinney 2007; Lidstrom and Konopka 2010; Walling and Shepard 2011), which makes the task of discerning the physiological status of a cell population using markers or reporters a real challenge. An additional layer of complexity is the further modulation of this phenotypic heterogeneity by environmental inputs.

It is thought that microorganisms may sometimes enter into a VBNC state as a survival strategy in response to unfavorable growth conditions or stressful environmental assaults. The term VBNC was coined by Colwell and collaborators in their early studies on the detection of *Escherichia coli* and *V. cholerae* in aquatic environments, in which they noted the importance to public health of measuring the true viable counts of water-borne pathogens (reviewed in Colwell 2000). They observed a markedly smaller number of culturable cells, compared to direct enumeration by microscopy-based techniques, suggesting the presence of subpopulations of cells that were non-culturable using standard culture methods yet could potentially serve as a reservoir of organisms retaining pathogenic potential (Colwell 2000). In the ensuing years, many non-sporulating bacterial species, including some pathogenic species, have been shown to form VBNC cells. Typically, the VBNC state is induced by exposure of bacteria to nutrient starvation or physicochemical stresses, resulting in diversified cellular phenotypes (Oliver 2010).

Traditional bulk-cell techniques, such as incorporation of radiolabeled precursor molecules (Rahman et al. 1994; Pawlowski et al. 2011), determination of intracellular ATP levels (Gengenbacher et al. 2010), real-time PCR (Asakura et al. 2006; Lahtinen et al. 2008; Lothigius et al. 2010; Trevors 2011), microarrays (Liu et al. 2008), and protein gel electrophoresis (Heim et al. 2002; Muela et al. 2008) can provide strong evidence of ongoing metabolic activity, including active transcription and translation (Table 1). However, these techniques are not optimal for the study of the VBNC phenomenon. Bulk-cell techniques provide population-averaged measurements, which do not necessarily provide reliable information about the status of individual cells or minor subpopulations (discussed in Dhar and McKinney 2007; Lidstrom and Konopka 2010; Walling and Shepard 2011; Yin and Marshall 2012). Thus, even the earliest studies of the VBNC state used bulk-cell techniques in conjunction with the single-cell techniques that were available at the time, especially epifluorescence microscopy and flow cytometry.

Total cell counts can be determined by epifluorescence microscopy of cells stained with dyes like acridine orange and 4′,6-diamidino-2-phenylindole, which stain nucleic acids (Roszak and Colwell 1987). Similarly, cellular "viability" can be assessed using microscopy-based techniques to measure cellular phenotypes that are thought to be characteristic of live or dead cells. Kogure's "direct viable counting" (DVC) method relies on the microscopic detection of cell elongation

Table 1 Old and new techniques for population-averaged and single-cell studies

Invasive bulk-averaged	Invasive single-cell	Live single-cell	Single-cell recovery
Radiolabel incorporation	Fluorescent dyes	GFP fusions	Flow cytometry
Intracellular ATP content	Fluorescence microscopy	MS2-tagging, PCA	FACS-based cell sorting
Real-time PCR	FISH	Imaging in microfluidics	Optical tweezers
Microarrays	RING-FISH	FLIP, FRAP	Dielectrophoresis (DEP)
Mass spectrometry	Flow cytometry	FRET	Microfluidic cell sorting

over time (Kogure et al. 1987); thus, it is not capable of distinguishing between dead cells and viable but non-growing cells. Respiratory activity of single cells can be detected by respiration-dependent reduction of 5-cyano-2,3-ditolyl tetrazolium chloride, a water-soluble non-fluorescent dye, to the corresponding formazan product, which is water-insoluble and red-fluorescent (Lew et al. 2010). Intracellular enzymatic activity can be detected by cellular uptake of non-fluorescent fluorescein diacetate (FDA) and its hydrolytic conversion to fluorescent FDA by intracellular esterases (Hamid Salim et al. 2006). Energization of the bacterial cytoplasmic membrane can be detected by staining cells with Rhodamine 123, a red-fluorescent dye that preferentially accumulates in energized membranes (Kell et al. 1998). Maintenance of the cell envelope permeability barrier can be assessed with the so-called "LIVE/DEAD" BacLight kit (Molecular Probes), which combines two nucleic acid stains: membrane-permeable SYTO9 (green) and membrane-impermeable propidium iodide (red), to identify "live" cells (which stain green) versus "dead" cells (which stain red).

Although these reagents can provide useful insights into the physiological status of single cells when used in conjunction with epifluorescence microscopy or flow cytometry (Davey et al. 2004), at least two drawbacks should be mentioned. First, the assays themselves are potentially toxic for bacterial cells, which may result in under-estimation of the number of "viable" cells in a population. Second, because the assays measure phenotypes that are only indirectly related to cell viability, they may result in incorrect assignment of cells to the "live" or "dead" category—for example, a cell that appears be dead because it can be stained with propidium iodide may nonetheless be capable of repairing its cell envelope and resuming active growth and division (Davey and Hexley 2011).

Fluorescent in situ hybridization (FISH) has also been used to study the VBNC phenomenon, especially the dynamics of fecal bacteria in river water. A combination of DVC and FISH has been used to assess the VBNC status of environmental *Helicobacter pylori*, in the presence or absence of different DNA-gyrase inhibitors (Piqueres et al. 2006), and Recognition of Individual Gene-FISH (RING-FISH) has been used to target low copy chromosomal DNA (Griffitt et al. 2011). However, one could argue that DNA is too stable to be a reliable indicator

of cell viability, and could potentially persist long after the cells had actually died; moreover, FISH requires sample fixation so it cannot be used on living cells (Table 1).

An important innovation allowing the real-time detection of cellular activities in unperturbed (unstained and unfixed) samples was the introduction of methods for engineering cells to express one or more chromatic variants of green fluorescent protein (GFP). By tagging cells with the expressed GFP marker, non-destructive measurements of single-cell fluorescence variation could be used to distinguish between different cell states—active, dormant, live, dead, VBNC, etc. (Lowder et al. 2000). The introduction of fluorescent expressed reporter tags opened a promising new avenue for the investigation of non-culturable cells that has only partly been exploited (Table 1).

2.2 Interpretations of the VBNC Phenomenon

In their natural environments, bacterial communities frequently encounter different types of stresses, such as nutrient starvation, natural antimicrobials, irradiation, temperature shifts, host immune mechanisms, and competition with other microorganisms. Besides genetic mutations, phenotypic heterogeneity due to non-genetic variability, arising from multiple sources such as stochastic fluctuations in gene expression and asymmetric partitioning of cell components during cell division, may allow microbes to adapt quickly to new conditions, guaranteeing the survival of at least a fraction of the original population (Balaban 2011; Davidson and Surette 2008; Dhar and McKinney 2007; Lidstrom and Konopka 2010; Walling and Shepard 2011). Not only single-cell variation in expression of specific genes that modulate the fitness of individual cells but also variation in single-cell growth rates could contribute to survival in fluctuating environments. Spontaneous entry of individual cells into a non-growing and potentially non-culturable state could potentially serve as an advantageous mechanism to attenuate or modify their metabolic requirements, thereby providing a long-term state of tolerance to multiple external stresses (Balaban et al. 2004; Kussell et al. 2005; Kussell and Leibler 2005).

An extreme example of an adaptive response to unfavorable conditions is the transition from replicating vegetative cell to non-replicating and metabolically dormant spore in sporulating bacteria. Spores are structurally distinct from vegetative cells, and the stimuli required for germination and outgrowth of spores are known. Recently it has been shown that the RNA profile is highly dynamic during and after the sporulation process, with the degradation rates of RNA molecules changing according to temperature variations (Segev et al. 2012). Amino acids and sugars are well-known germinants, and cell wall muropeptides released by growing cells have been shown to play a major role in exit from dormancy in *Bacillus subtilis* spores (Shah et al. 2008). Once triggered, germination is a fast process that is dependent on the ability of dormant spores to sense changes occurring in their external environment. The signals and processes that govern

sporulation and germination are understood in considerable detail. In contrast, the physiological basis and dynamics of entry into and exit from a state of non-culturability in non-sporulating bacteria, which may provide some of the same survival advantages as sporulation, are less well understood. In part, this "knowledge gap" stems from the difficulty of obtaining for analysis homogeneous populations of VBNC cells with no contaminating culturable cells (Barcina and Arana 2009).

By comparison, more is known about the ability of non-sporulating bacteria to sense changes in their external environment and to modulate their growth rate accordingly. For example, the stringent response is triggered by various stresses including nutrient (especially amino acids) starvation, leading to intracellular accumulation of the "alarmone" (p)ppGpp, which influences both initiation and elongation of rRNA synthesis and expression of stress-response genes (Jin et al. 2012). Another phenomenon observed in several bacterial species during stationary phase is the so-called "ribosomal hibernation", which is controlled by a set of proteins that promote the transition and dimerization of the active 70S ribosomal subunit into the inactive 100S form. Formation of 100S hibernating ribosomes is a mechanism used by both Gram-negative and Gram-positive bacteria to temporarily interrupt the ribosome cycle under stressful conditions (Ueta et al. 2008, 2010; Williamson et al. 2012).

In this chapter, we will focus on phenotypic states of non-culturability in which the cells retain metabolic activities that can be detected at the single-cell level using a variety of techniques, some of which have already been described. Because the viability (i.e., future replicative potential) of these cells is uncertain, we will largely eschew the term VBNC, which seems to beg the question. As an alternative, we propose the more conservative term NGMA to describe cells in this physiological state, which are indeed something of an *enigma* in the field of microbiology.

The search for stimuli and molecular mechanisms that could trigger entry into and exit from the NGMA state has elicited different interpretations from different groups. Nyström interpreted the phenomenon of bacterial non-culturability, induced by starvation or stress, in two alternative ways (Nyström 2001). The first interpretation evokes direct causation, whereby loss of culturability could be due to cellular deterioration after exposure to essentially any damaging stress. Although metabolic rates are reduced under nutrient starvation, the respiration, and consequent production of reactive oxygen species (ROS) probably remain substantial, as suggested by the observed induction of oxidative stress-response proteins and accumulation of oxidatively-damaged proteins (Nyström 2001; Desnues et al. 2003). Consistent with the idea that ROS might contribute to the non-culturability of nutrient-starved cells, it has been shown that addition of catalase or ROS scavengers to the medium of nutrient-starved cultures increased plating efficiency (i.e., culturability) of the stressed cells (Mizunoe et al. 1999). The second interpretation evokes indirect causation, whereby an active adaptive response allows stressed cells to enter into a non-replicating and non-culturable state as a mechanism to promote their long-term survival in the face of potentially

lethal stresses. Consistent with the idea that non-culturability is an actively regulated phenomenon, mutants of *V. cholera* have been isolated that display altered frequencies of entry into the VBNC state (discussed in Nyström 2001). Stress could also arise from a natural process of self-catabolism during long-term nutrient starvation, resulting in depletion of intracellular stores and cellular components and gradual accumulation of intracellular waste products. The ability of subpopulations of aging cells, during prolonged stationary phase, to express stress-response genes and outcompete younger cells was defined as a "Growth Advantage in Stationary Phase" (GASP) phenotype (Finkel 2006; Navarro Llorens et al. 2010). GASP was proven to be due to accumulation of advantageous mutations in aging cultures, thereby increasing the overall fitness of the population under nutrient-limiting conditions.

Focusing on these stages of life in stationary-phase bacterial communities: on the one hand, the high-density population of nutrient-scavenging cells leads to nutrient depletion from the external milieu; on the other hand, death of a fraction of cells releases molecules that can be scavenged by the survivors. The simultaneous occurrence of these two opposing processes could permit the maintenance of a relatively stable number of culturable cells over time due to dynamic and balanced processes of cell death and cell proliferation. It could even be envisaged that natural selection might favor a "division of labor" along these lines, in which starving populations bifurcate into cells that are "eaters" and cells that are "eaten." Consistent with this idea, a model of cannibalism has been reported for *B. subtilis* in which starving populations show marked cell-to-cell variation in the expression of the master regulator Spo0A, which governs exit from vegetative growth and entry into the differentiation pathway leading to spore formation. Individual cells with high levels of Spo0A produce toxins that kill and lyse their non-sporulating siblings, thereby releasing nutrients that delay commitment to sporulation (Gonzàlez-Pastor 2011).

Connecting this concept to the NGMA phenomenon, any bacterial population subjected to environmental challenge or nutrient limitation could potentially release molecules, either to kill or to cause loss of culturability in other members of the population, thereby reducing the overall metabolic rates and nutrient requirements of the population. The presence of so-called "altruistic" and "survivor" members within a bacterial population has been proposed as a successful survival strategy to cope with environmental stresses (Barcina and Arana 2009). The maintenance of "non-demanding" subpopulations of cells could represent an inexpensive bacterial reservoir for future re-expansion when auspicious conditions return. Moreover, slowing down or freezing cellular functions could reduce the physiological impact of target inhibition by antimicrobials.

The "Scout Hypothesis" provides a different perspective in the presence of apparently altruistic cells in a dormant bacterial population (Epstein 2009). According to this hypothesis, individual cells within a dormant population could periodically "wake up" due to stochastic changes in expression of a master regulatory gene, for example. The awakened "scout" cells may resume proliferating if conditions are favorable or may die if conditions are adverse. Such random

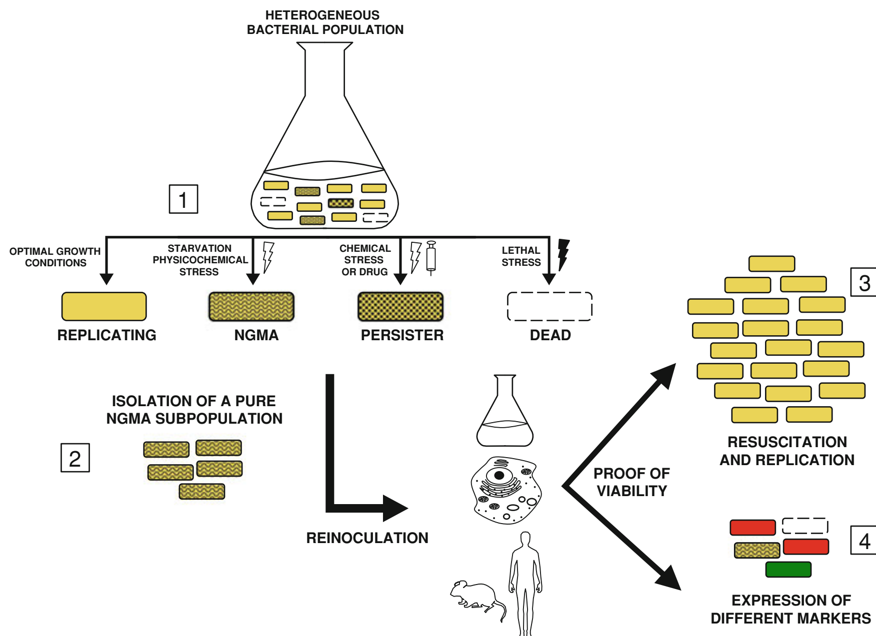

Fig. 2 Bacterial heterogeneity and identification of NGMA subpopulations. Bacterial populations are characterized by the presence of physiologically distinct subsets of cells, due to genetic and non-genetic heterogeneity, dynamic patterns of gene expression, cell–cell communication, environmental fluctuations, and physical stresses (*1*). The classical method of NGMA cells isolation by dilution to extinction of the replicating populations and reinoculation in vitro, ex vivo, or in vivo is commonly used to demonstrate viability through resuscitation of the non-culturable population (*2* and *3*). The use of fluorescent reporter-tagging methods, coupled to cell sorting, optical tweezers, or dielectrophoresis (DEP), represents a valid alternative approach to isolate and purify NGMA supopulations, which can then be subjected to 'omic studies or reinoculated under different environmental conditions to test for resuscitation (*4*)

awakenings have been proposed as a parsimonious strategy for a population of dormant cells to sample the environment, thereby risking only a few individuals to sense the environment and stimulate the revival of their dormant siblings if conditions are favorable for growth. Recently, the scout hypothesis has been investigated in *E. coli* and *Mycobacterium smegmatis*, a non-pathogenic and fast-growing relative of *M. tuberculosis* (Buerger et al. 2012).

The common element linking these studies and interpretations is that bacterial populations are heterogeneous, comprising subsets of cells characterized by different phenotypes and different roles within their community. Long-term single-cell studies are required to better understand the NGMA phenomenon, and to elucidate how non-genetic phenotypic diversity within a population could potentially influence the response and survival of bacterial populations under different stressful conditions (Fig. 2).

2.3 Recovery and Virulence of Non-Culturable Cells

A common challenge in the VBNC field is to perform successful resuscitation experiments, thereby proving that revival is truly due to resuscitation of previously non-culturable cells rather than outgrowth of a small minority of viable and culturable cells still present in the population. This technical problem highlights once again the need for new approaches to obtain pure subpopulations of bacteria from mixed cultures (Fig. 2).

A commonly used method utilized to separate culturable from non-culturable cells is the Most Probable Number (MPN) strategy, which involves serially diluting a mixed culture sufficiently such that each subculture contains, on average, less than one CFU (Roszak and Colwell 1987). Afterward, resuscitation of the diluted cultures is induced by appropriately modifying the culture conditions and the appearance of culturable cells is monitored using appropriate techniques, for example, by counting CFU. If the number of viable counts that appears in the stimulated cultures exceeds the number that could be achieved by simple proliferation of a small number of culturable cells contaminating the original culture, the experimenter can infer that previously non-culturable cells have been resuscitated (i.e., have reverted to a culturable state) by the stimulus.

Experiments with *Micrococcus luteus* have provided one of the most convincing cases of bacterial resuscitation from a state of non-culturable dormancy (Kaprelyants and Kell 1993). Transition of *M. luteus* to a dormant state can be induced by prolonged incubation in spent culture medium. Typically, following entry into stationary phase, the CFU count declines to non-detectable levels while the total cell count decreases by about 40 % and then stabilizes. Entry of cells into the non-culturable state is accompanied by a reduction in cell diameter, respiration rate, rhodamine uptake, and protein amount per cell, whereas the DNA content per cell remains constant. To eliminate any residual culturable cells, bacteria are further treated with penicillin G, which kills replicating cells but spares non-replicating cells. Resuscitation is then triggered by washing the dormant cells and inoculating them into fresh medium at high density. About 20 h later, the revived bacteria show signs of metabolic activity and the viable counts return to the initial level. The increase in CFU following resuscitation can be ascribed to resuscitation of previously non-culturable cells, because the 4-h generation time of *M. luteus* is too long to explain the observed increase in CFU from 1,000 CFU/ml at 30 h of recovery up to 100 million CFU/ml at 50 h. However, expansion of the CFU count by this magnitude could be explained by re-awakening of a large fraction of a VBNC population whose starting total cell number, based on microscopic counts, was about 200 million CFU/ml.

Interestingly, resuscitation of non-culturable cells of *M. luteus* can be enhanced by addition of cell-free supernatant from stationary-phase cultures (Kaprelyants et al. 1994). The extracellular factor responsible for recovery of dormant *M. luteus* has been identified as a secreted protein termed Resuscitation Promoting Factor

(Rpf). Purified *M. luteus* Rpf can also exert a resuscitation effect on heterologous species, including *M. tuberculosis*. It is noteworthy that the *M. tuberculosis* genome encodes five Rpf-like genes (Mukamolova et al. 2002), which are collectively dispensable for growth in liquid medium but required for resuscitation from a non-culturable state; interestingly, this defect can be rescued by addition of wild-type culture supernatant (Kana et al. 2008).

Taking into consideration the evidence suggesting that non-culturable cells of different species can be resuscitated by appropriate stimuli, an important question is the state of virulence of these non-replicating populations. This problem is relevant not only for water-borne pathogens, which have historically been a major focus in the VBNC field, but also for food-borne or air-borne bacteria that cause long-term clinically inapparent infections, such as *M. tuberculosis*. To test the virulence potential of VBNC cells, it is first required to isolate pure sub-populations of non-culturable cells, assess their potential to be resuscitated, and verify the ability to re-initiate an infection (Fig. 2). This procedure in fact recalls Koch's postulates, four criteria proposed to establish a deterministic relation between a disease and its causative microbe. Once a microorganism has been identified and isolated as a pure culture from a diseased host, it should be able to cause the same disease if re-inoculated into a healthy host; it should then be possible to re-isolate the microorganism from the secondarily infected host and confirm that it is identical to the initial causative agent. Even if we accept the concept that in vivo resuscitation of non-culturable bacteria represents a potential risk to human health, it will be difficult to demonstrate that resuscitation and virulence are exclusively due to the reawakening of non-culturable cells, unless and until a clean procedure for single-cell identification and purification becomes available.

In 1995, Oliver and Bokian tested whether non-culturable cells of *Vibrio vulnificus* were able to infect mice (Oliver and Bockian 1995). The VBNC state was induced by incubation of *V. vulnificus* in artificial seawater and exposure to a temperature downshift, causing the viable counts in the culture to drop to levels that were undetectable by Kogure's method. Subsequently, mice were infected with different doses of the VBNC culture and the lethal dose causing death of half the infected mice (LD_{50}) was determined, as well as viable counts (CFU) from various tissues. These experiments established that VBNC cells of *V. vulnificus* retain pathogenicity. However, there was an inverse temporal relationship between time of incubation at low temperature and virulence, which could indicate either a gradual shift into the VBNC state, the presence of mixed populations of culturable and non-culturable cells in the inocula used to infect the animals, or gradual death of VBNC cells.

A similar example of in vivo resuscitation was performed in human volunteers using an attenuated strain of *V. cholerae* O1 that expressed a mutated subunit of cholera toxin (Colwell et al. 1996). The non-culturable state was induced by nutrient starvation and temperature downshift for either 10 or 28 days; total and viable (culturable) counts were measured using standard techniques. Each volunteer ingested about one billion cells, including an estimated ten million VBNC

cells, the remainder being simply non-viable. Remarkably, "young" (10 days) inocula of VBNC cells retained the ability to cause infection resulting in clinical signs and symptoms, whereas "old" (28 days) inocula did not.

Interpretation of studies reporting in vivo resuscitation and virulence of VBNC cells, whether in mice (Oliver and Bockian 1995) or in humans (Colwell et al. 1996), is complicated by the difficulty of achieving pure VBNC populations using standard bulk methods. Interestingly, VBNC cells of several species have been shown to exit the non-culturable state when co-cultured with different types of eukaryotic cells (Senoh et al. 2012). Further studies along these lines would greatly benefit from the advent of new non-destructive single-cell techniques to isolate pure subpopulations of non-culturable cells in order to assess the pathogenic potential of non-culturable bacteria both in vivo and ex vivo.

3 *Mycobacterium tuberculosis*

3.1 *Mycobacterium tuberculosis: A Case of Dormancy and Revival*

Bacterial growth is a complex process that can display a broad spectrum of states. In comparison to actively replicating cells, which are the usual object of microbiology research, assessing the physiological states of non-replicating cells can be technically challenging. Dormancy, a reversible state of low metabolic activity used by non-sporulating bacteria to survive for long periods of time under stressful environmental conditions, is often coupled to non-culturability.

M. tuberculosis is a major human pathogen and a prominent example of bacterial dormancy. It is estimated that perhaps one-third of the global human population is latently infected with tubercular bacilli, and it is generally assumed (although not proven) that these bacteria are metabolically sluggish or quiescent. Understanding latent *M. tuberculosis* infection is important for at least two reasons: first, reactivation of latent *M. tuberculosis* infections represents a huge burden of future disease; second, latent *M. tuberculosis* infections are thought to be refractory to antibiotic therapy (Young et al. 2006). Regarding the latter, there is an apparent discrepancy between the refractoriness of nonreplicating *M. tuberculosis* toward drugs that target cell wall biogenesis, such as isoniazid, and the effectiveness of prolonged prophylactic isoniazid therapy in preventing reactivation of latent tuberculosis infection. This discrepancy could indicate that dormant cells of *M. tuberculosis* retain some sensitivity to wall-targeting antibiotics or, alternatively, that dormant cells periodically "wake up" and revert to an active state of drug susceptibility. A related question concerns the true extent of effectiveness of anti-tuberculosis therapy for active disease, and whether some dormant bacteria could persist in the lungs despite prolonged dug therapy and maintain the

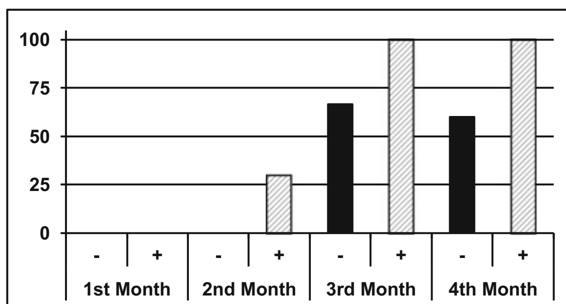

Fig. 3 Percentage of animals exhibiting relapse, after prolonged treatment with combination of drugs. Cortisone was administered (+) in groups of mice, which were monitored over a period of 4 months following anti-tuberculosis drug therapy (Modified from McCune et al. 1966)

potential to reactivate in the future. These open questions underscore the need to elucidate the metabolic state of nonculturable *M. tuberculosis*, a topic that had been investigated intensively over the years but which has not, as yet, yielded much in the way of definitive answers.

In the 1950s and 1960s, McCune and colleagues at Cornell University Medical College performed a series of pioneering experiments focused on *M. tuberculosis* dormancy and antibiotic persistence in a murine model of tuberculosis (McCune et al. 1966). In the so-called "Cornell Model", mice were infected with *M. tuberculosis* and treated extensively with various combinations of anti-tuberculosis drugs. At the conclusion of treatment, the bacilli were apparently cleared ("vanished") from the lungs of mice that received the most effective regimens, inasmuch as no remaining bacteria could be detected using sensitive techniques, such as CFU assays, acid-fast staining of lung sections, and re-inoculation of tissue homogenates into normal and immune-suppressed mice. Nonetheless, it became clear that the apparently "cured" mice still harbored tubercle bacilli, apparently in a non-culturable state, because immune suppression of these animals led to nearly uniform bacteriological relapse, as verified by CFU counts of tissues following immune suppression (Fig. 3). Remarkably, the bacteria that were recovered by plate culture post-relapse retained sensitivity to the drugs that were used to treat the infected animals. In sum, prolonged treatment of infected mice with anti-tuberculosis drugs resulted in "vanishing" of culturable bacteria from the tissues, whilst immune-suppression of the treated animals led to "reappearance" of culturable bacteria in the tissues. These experiments provided strong evidence for reactivation of *M. tuberculosis* from a VBNC-like state in vivo. Similar results have been reported in a guinea pig model of tuberculosis (Obregón-Henao et al. 2012).

Molecular analyses of the "vanishing" phenomenon in the Cornell Model have also been performed (de Wit et al. 1995; Hu et al. 2000). De Wit and colleagues searched for the presence of *M. tuberculosis* DNA in tissue homogenates using quantitative PCR (qPCR). The estimated bacterial count by qPCR in acutely infected mice was similar to the numbers detected by plating for CFU, whereas in

chronically infected mice the bacillary count by qPCR exceeded the CFU count, suggesting that some bacteria had died, or, alternatively, entered into a VBNC-like state. Following prolonged drug therapy of infected mice, resulting in the "vanishing" of CFU from the tissues, bacterial DNA could still be detected in the tissues by qPCR; however, this approach was not informative about the metabolic status and potential for resuscitation of bacteria in the tissues of the treated animals and the detected DNA could simply represent dead cells. Inoculation of the apparently sterile (no CFU) tissue homogenates into healthy mice resulted in the induction of infection and disease in a small fraction of the inoculated animals. In these experiments, as in the original experiments by the Cornell group, it is extremely difficult to distinguish between resuscitation of VBNC cells and outgrowth of a very small number of persistent viable (culturable) cells that may have escaped detection. In principle, these interpretations could be distinguished by purification of the nonculturable cell population in order to exclude any contamination by culturable cells prior to inoculation. In practice, this ideal goal seems unachievable using current techniques.

In principle, the relatively shorter half-life of RNA compared to DNA should make RNA a better marker of the metabolic state of dormant and VBNC-like *M. tuberculosis*. Hu et al. (2000) detected the presence of ribosomal RNA and messenger RNA transcripts in the Cornell Model and in an in vitro model of hypoxia-induced dormancy, the so-called "Wayne model" (Wayne 1976). They also demonstrated ongoing transcription in rifampicin-treated cultures of *M. tuberculosis* in vitro by measuring incorporation of tritiated uridine, despite the "vanishing" of detectable CFU in the rifampicin-treated cultures (Hu et al. 2000). In these experiments, it was also shown that inoculation of liquid medium with serially diluted cultures provided a more sensitive measure of residual viable counts compared to inoculation of solid medium and CFU formation. A similar discrepancy between results obtained by inoculation of liquid versus solid media was also reported for experiments with stationary-phase cultures or tissue homogenates from chronically infected mice (Dhillon et al. 2004). These observations underscore the fact that the current distinction between viable and VBNC-like mycobacteria is essentially an operational definition (does the cell form a colony on solid medium? does it replicate in liquid medium?) that provides little insight into the actual physiology of these two contrasting states.

Despite the pioneering work done by the Cornell group nearly half a century ago, in the intervening decades there has been rather slight progress in the understanding of the VBNC-like state in *M. tuberculosis*. The relative stagnation of this area of inquiry, which lags behind the remarkable advances that have been made in other areas of tuberculosis research in recent years, underscores the lack of suitable tools to query the physiology of VBNC-like cells of *M. tuberculosis* at the single-cell level in the absence of small numbers of contaminating and potentially confounding "ordinary" (i.e., viable and culturable) cells.

3.2 Resuscitation in Mycobacteria: From Bulk to Single-Cell Studies

The physiological and molecular bases of mycobacterial entry into and exit from a state of dormancy are still poorly understood, despite the existence of several models in vitro (Hu et al. 2000; Dhillon et al. 2004; Sala et al. 2010; Shleeva et al. 2011), ex vivo (Biketov et al. 2000), and in vivo (McCune et al. 1966; Obregón-Henao et al. 2012). In this section, we will focus on experiments that have attempted to address these questions, and in this context we will argue for the potential importance of single-cell approaches based on new technologies and nondestructive techniques that permit the real-time tracking of individual cells.

Long-term in vitro cultures subjected to medium acidification, oxygen depletion, or nitric oxide exposure are some of the conditions that have been used to induce *M. tuberculosis* dormancy, based on the belief that these conditions mimic the conditions that mycobacteria encounter in vivo in the host tissue environment. After prolonged incubation of *M. tuberculosis* in stationary-phase liquid cultures, the cells become nonculturable and phase-dark ovoid cell bodies accumulate (Shleeva et al. 2011). These morphologically abnormal cells are dwarfish, acid-fast positive, and propidium iodide negative, implying an intact cell envelope barrier; although these cultures are non-culturable in the sense that they do not form colonies on plates, they incorporate tritiated uracil and they are capable of outgrowth when inoculated into liquid medium. Furthermore, using the MPN method described above, these non-culturable cells can be resuscitated by exposure to culture supernatants from actively growing cultures of *M. tuberculosis* or (albeit less efficiently) by exposure to Rpf from *M. luteus* (Shleeva et al. 2011). Similar results have been obtained in experiments demonstrating the ability of phospholipids and peptides isolated from culture supernatants of *M. tuberculosis* to resuscitate very old (6 months) stationary-phase cultures of *M. tuberculosis* (Zhang et al. 2001). Fatty acids, especially oleic acid, can also promote the resuscitation of VBNC-like cells of *M. smegmatis* (Nazarova et al. 2011). It has also been shown that *M. tuberculosis* in the sputum of untreated patients could be cultivated only in the presence of Rpf; however, this Rpf-dependence was eventually lost after sustained in vitro cultivation (Mukamolova et al. 2010). Rpf displays peptidoglycan hydrolytic activity, and it has recently been shown that mycobacterial peptidoglycan fragments, obtained by Rpf hydrolysis, are sufficient to resuscitate VBNC-like mycobacterial cells (Nikitushkin et al. 2013).

The presence of non-culturable forms of *M. tuberculosis* has also been inferred from in vivo studies using a “replication clock” system to assess the replication dynamics of *M. tuberculosis* in chronically infected mice (Gill et al. 2009). This system comprises a strain of *M. tuberculosis* harboring an unstable episomal plasmid that is lost at a rate proportional to the rate of bacterial division. By measuring the rates of plasmid loss during the course of murine infection, in

conjunction with mathematical modeling, the authors concluded that the actual numbers of division events occurring in chronically infected mice exceeded the numbers predicted by counting CFUs. These observations suggest that a fraction of the replicating population is either killed or enters into a VBNC-like state, thereby resulting in a deceptively stable number of culturable cells (CFU) over time.

Although the experiments described above provide evidence for the existence of VBNC-like forms of *M. tuberculosis* that can be resuscitated by appropriate stimuli, the conclusions are largely based on indirect assays, such as MPN, and are limited to bulk culture measurements or destructive microscopy techniques. Thus, they provide little insight into the physiology and behavior of individual cells during entry into and exit from the VBNC-like state. Flow cytometry, combined with fluorescent markers and dyes that can report on specific aspects of bacterial physiology—such as gene expression, respiratory activity, membrane potential, etc.—can provide detailed insight into the "instantaneous" phenotype of individual cells (Davey et al. 2004). A major drawback of this technique, however, is that it does not permit the temporal tracking of individual cells and, unless coupled to cell sorting, each cell that is measured is lost to further analysis. Thus, flow cytometry suffers from the same limitation as the techniques described above, viz. it cannot be used to track the behavior of individual cells as they enter into and exit from the VBNC-like state.

A promising technique to separate and recover subpopulations of cells for further analysis is cellular dielectrophoresis (DEP), which can be used to separate intact and damaged cells based on their different dielectric behavior (Table 1). Recently, DEP has been used to study non-culturable populations of *M. smegmatis* and to separate live, dead, and dormant cells (Zhu et al. 2010a, b). In these studies, the authors used exponentially growing, late stationary-phase, and killed cultures of *M. smegmatis*. Each of these cell preparations was characterized by differences in cell morphology, structure, and composition, resulting in different cellular conductivities, which is a *sine qua non* condition for DEP-based separation of dielectric particles. In particular, the calculated conductivities were highest for dead cells (915 ± 15 μS/cm), intermediate for stationary-phase cells (812 ± 10 μS/cm), and lowest for exponentially growing cells (560 ± 20 μS/cm), presumably due, at least in part, to differences in the insulating properties of the cell envelope. By modulating the frequency of the alternating current electric field, crossover frequencies were identified at which cells with different conductivities displayed either positive DEP (particles migrate to regions of high field strength) or negative DEP (particles migrate to regions of low field strength) (Zhu et al. 2010b). Thus, DEP-based separation is a potentially powerful approach to obtain relatively pure preparations of cell subpopulations from mixed cell cultures; if this technique can be scaled up, it might be possible to obtain sufficient numbers of purified cells to perform detailed phenotypic analyses of these distinct cell states. An important caveat is the damage that could result from subjecting cells, even briefly, to a strong electric field, which could lead to damage-related artifacts.

Notwithstanding this potential confounder, DEP-based cell separation looks like a promising approach to purification and analysis of bacterial subpopulations from mixed starting cultures.

4 Tools to Investigate the NGMA Phenomenon at the Single-Cell Level

Microbial phenotypic heterogeneity occurs at different levels of environmental complexity, ranging from axenic cultures in vitro to host tissue environments in vivo, where fluctuations of physico-chemical parameters can induce replicating cells to die or enter a non-replicative state while maintaining a certain level of metabolic activity. A deeper understanding of the NGMA phenomenon at the single-cell level—and, in particular, the ability to track individual cells as they enter and exit the NGMA state—would provide new insights into the survival mechanisms of microorganisms. The metabolism and physiology of individual cells, linked to different growth states (e.g., replicating, non-replicating, and non-culturable), can be characterized by measuring fundamental cellular activities at the single-cell level, such as growth, division, DNA replication, gene expression, biochemical networks, respiratory activity, membrane energization, metabolite fluctuations, etc. (Fig. 1).

Phenotypic heterogeneity can arise from multiple sources, including (but not limited to) stochastic flucutations in gene expression (Golding et al. 2005; Yu et al. 2006; Larson et al. 2009; Suter et al. 2011; Ferguson et al. 2012; Lionnet and Singer 2012), noisy production of proteins (Cai et al. 2006; Bar-Even et al. 2006), and asymmetric partitioning of cell components at division (Huh and Paulsson 2011). A number of studies have demonstrated that both eukarytic and prokaryotic cells are characterized by extremely heterogeneous dynamics in gene expression. These processes can be studied at the single-cell and single-gene level by use of fluorescent reporters, time-lapse microscopy, and quantitative modeling tools.

Two broad categories of gene expression noise have been proposed to influence the transcriptional activity of individual genes (Locke and Elowitz 2009; Lionnet and Singer 2012). “Extrinsic” noise reflects fluctuations at the level of the cell; for example, time-dependent variation in the amount or activity of a regulatory factor. “Intrinsic” noise reflects fluctuations at the level of the gene itself; for example, thermodynamic fluctuations affecting the binding or unbinding of a regulatory factor to its cognate binding site on the DNA. These two sources of gene expression noise obviously intersect, as, for example, intrinsic noise affecting the expression of a regulatory factor could propagate as extrinsic noise affecting expression of the target genes that are regulated by that transcription factor.

It is conceivable that entry into and exit from the NGMA state could be triggered, or at least modulated, by noise-driven fluctuations in gene expression,

resulting in transient phenotypic switching between growing and non-growing states. This is an area of investigation that seems ideally suited to real-time, single-cell experimental approaches, such as time-lapse fluorescence microscopy, in conjunction with computational modeling (Locke and Elowitz 2009; Chubb and Liverpool 2010). Within this realm, there is broad scope for application of the rapidly expanding arsenal of biomolecular and microengineered technologies (Rowat, et al. 2009; Tang et al. 2009; Taniguchi et al. 2010; Dalerba et al. 2011; Fidalgo and Maerkl 2011; Gobaa et al. 2011; White et al. 2011; Srigunapalan et al. 2012). For example, highly automated microfluidic tools, micro-patterned with chambers to contain cells, microchannels for delivery of liquid media, and pneumatic valves to permit user-specified opening and closing of the microchannels, allow rapid and precise control over the cells' microenvironment. These microdevices can be deigned and inexpensively manufactured to match the end-users' specific requirements and can be used in conjunction with time-lapse microscopy for real-time single-cell analysis of microbial responses to fluctuating environments.

The use of fluorescent reporter strains of bacteria, often comprising fusions of sequences encoding a fluorescent protein to promoter sequences (transcriptional fusions) or protein-coding sequences (translational fusions), has revolutionized the study of gene expression in single cells. This approach is particularly powerful when combined with live-cell time-lapse microscopy and microfluidic culture systems that provide dynamic control over the culture environment (Golding et al. 2005; Lionnet and Singer 2012). The development of new fluorescent proteins, such as short-lived (Andersen et al. 1998), "timer" (Terskikh et al. 2000), photoactivatable (Patterson and Lippincott-Schwartz 2002), and photo-switchable variants (Bates et al. 2007), is progressively expanding the spectrum of applications and enabling deeper insights into intracellular processes, time-dependent gene expression patterns, protein localization in space and time, and molecular interactions at the sub-micron scale (Table 1).

In the specific case of the NGMA phenomenon, there is exciting but largely untapped potential in the use of transcriptional and translational fusions of fluorescent proteins to genes controlling processes involved in cell growth, metabolism, ribosome production, chromosome replication, etc., to elucidate the physiology of individual NGMA cells (Fig. 1). By tracking time-dependent changes in the production, degradation, and localization of fluorescently-tagged proteins in single cells, it should be possible to construct a picture of the metabolic activities and potentialities of NGMA cells as compared to replicating cells. The use of conditional gene expression systems to switch on/off the expression of fluorescent reporters should also permit a quantitative analysis of the transcriptional and translational potentialities of NGMA cells compared to replicating cells. Demonstration that individual NGMA cells retain the ability to respond to an external inducing agent by switching on de novo transcription and translation of a fluorescent reporter would go a long way to strengthening the case that these cells might be viable.

New tools have also been introduced for microscopy-based monitoring of time-dependent fluctuations in single-cell gene expression at single-molecule resolution. Examples include the mRNA MS2 tagging system (Golding et al. 2005) and the fluorescent Protein Complementation Assay (PCA) (Valencia-Burton et al. 2007). These techniques can be used to monitor individual gene transcription events in real-time, as a stretch of the newly transcribed RNA, tagged with MS2 arrays or by RNA aptamers (which are bound by copies of split GFP that combine only at the moment of transcription), provide very fine temporal resolution of individual gene expression events (Table 1).

Other relevant methods that could be appropriate for investigating the physiology of individual NGMA cells include the combination of fluorescent reporters with photobleaching techniques, optical nanosensors, and real-time fluorescence microscopy. These tools can be used for single-cell techniques such as Fluorescence Loss in Photobleaching (FLIP), Fluorescence Recovery after Photobleaching (FRAP), and nanosensors based on Förster Resonance Enegy Transfer (FRET) (Lippincott-Schwartz et al. 2001). In both FLIP and FRAP, PB causes the loss of fluorescence in one portion of a cell or the entire cell. In FLIP, the intracellular movement of molecules can be followed through fluorescence diffusion after PB. In FRAP, the resurgence of fluorescence provides a real-time readout of de novo transcription and translation occurring after PB. FRET-based nanosensors employ two distinct fluorescent proteins, a donor molecule and an acceptor molecule, characterized by overlapping emission spectra and coupled to a substrate-binding protein domain. Upon binding of the specific substrate to the substrate-binding protein, conformational changes bring the fluorescent proteins into closer juxtaposition, thereby enhancing resonance energy transfer from donor to acceptor following excitation of the donor. Thus, changes in the fluorescence ratio (donor fluorescence : acceptor fluorescence) provide an estimate of the intracellular substrate/metabolite concentration.

Combining the aforementioned techniques with flow cytometry-based cell sorting, microfluidic cell sorting, DEP-based cell sorting (Zhu et al. 2010a), or single-cell trapping with optical tweezers (Ericsson et al. 2000) could, in principle, allow the isolation and collection of NGMA cells for 'omics-based studies, new targets identification, and virulence assessment through in vivo recovery experiments (Fig. 2). Fluorescent reporter strains can also be used for live-cell studies of host-pathogen interactions, thereby avoiding the artifacts associated with cell fixation (Fig. 4). Microfluidic devices for ex vivo studies (James et al. 2009), in vivo imaging systems (Manicassamy et al. 2010; Nam et al. 2011), and intravital microscopy (Meissner et al. 2009) have become increasingly available to academic researchers. Despite these exciting advances, in vivo imaging is still far from achieving the sensitivity and resolution required to perform studies on individual bacteria residing within infected animals. In principle, infecting cultured mammalian cells or animal models with bacterial reporter strains whose intracellular activities can be monitored through a wide variety of fluorescent reporters would provide the most direct way to visualize growth dynamics, metabolic activity, cellular processes, and virulence potential of both replicating and NGMA bacteria.

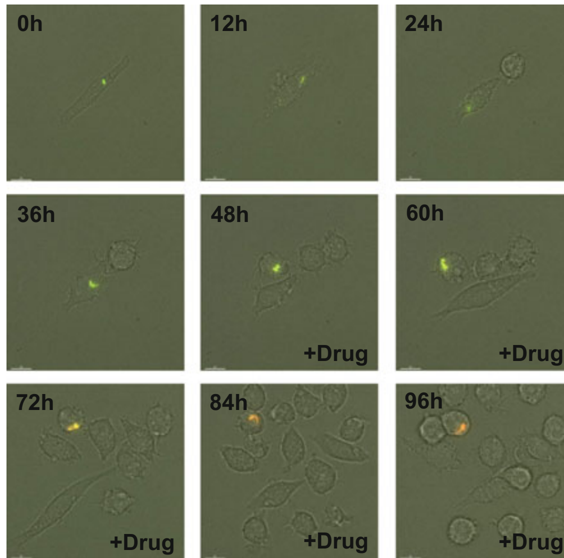

Fig. 4 Microscopy-based real-time analysis of host-pathogen interactions at the single-cell level. RAW macrophages infected with two-color fluorescently tagged *M. tuberculosis* (*GFP* short-lived reporter of metabolic activity, *DsRed* long-lived constitutively expressed reporter). Representative snapshots were taken over 4 days of continuous time-lapse fluorescence microscopy of a single macrophage infected with a single bacterium. The addition of a potent anti-tuberculosis drug to the medium causes a marked decrease in green fluorescence (metabolic reporter) within 24 h

5 Conclusions

The ecological and clinical relevance of VBNC bacteria has been a fascinating and prominent but largely unresolved issue in microbiology for several decades. Due to limitations in standard culturing techniques, artifacts associated with cell fixation, and potential toxicity of reporter dyes, there is a clear need for new methods to study the physiology of non-growing but physically intact and metabolically active bacteria. Ideally, such studies should be focused on the single-cell level. Population-averaged bulk-cell measurements can provide only partial and potentially misleading information on NGMA cells, inasmuch as there is no certainty of isolating pure subpopulations of NGMA cells, which might themselves comprise multiple distinct phenotypic states. Moreover, the standard limiting dilution techniques used to isolate non-growing from growing cells has obvious limitations and resuscitation experiments would benefit from higher-resolution techniques. A promising approach that has not been fully exploited to date is the combination of fluorescent reporter strains, time-lapse fluorescence microscopy, and microfluidic systems for real-time single-cell analysis of NGMA cells. Other techniques for single-cell analysis, some of which have been described in this chapter, have

proven their value in single-cell analysis of replicating cells but have not, so far, been applied to the analysis of NGMA cells. Tools to detect and measure molecular events and dynamics within intact and metabolically active cells that have lost (at least temporarily) the ability to grow and divide offer alternative ways to assess cellular "viability". For example, detection of ongoing transcription, translation, and metabolic activity (e.g., ATP synthesis) within individual non-growing cells would provide compelling evidence that these cells retain fundamental activities associated with "viability" and may also retain the capability of reactivating growth if provided with the appropriate stimulus.

In the specific case of *M. tuberculosis*, there is considerable scope for applying new single-cell approaches to the study of dormant and persistent states in this unusually refractory pathogen. *M. tuberculosis* is considered to be an exemplar of "persistent pathogens" that maintain long-term parasitic relationships with their chronically infected mammalian hosts by slowing their rates of metabolism, growth, and cell division. Reactivation of *M. tuberculosis* after years or even decades of clinically latent infection may, in some respects, resemble the resuscitation of non-culturable microorganisms. The application of new single-cell micro-technologies to the study of mycobacteria is still in its infancy (Aldridge et al. 2012; Golchin et al. 2012; Wakamoto et al. 2013), and to the best of our knowledge the new approaches have not been applied to the specific problem of the NGMA state in mycobacteria. In future, it should be possible to develop co-culture microsystems for real-time single-cell analysis of host-pathogen interactions—for example, bacteria interacting with cultured host cells or bacteria within tissue sections derived from infected animals. These approaches could provide new insights into the NGMA phenomenon during the course of infection and might identify new strategies for targeting this physiologically distinct subpopulation.

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