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Single-cell reporters for pathogen responses to antimicrobial host attacks

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Host–pathogen interactions are often heterogeneous involving individual encounters between host and pathogen cells with diverse molecular mechanisms, response networks, and diverging outcomes. Single-cell reporters can identify the various types of interactions and participating pathogen subsets, help to unravel underlying molecular mechanism, and determine individual outcomes and their impact on disease progression. In this review, we discuss reporters-based on fluorescent proteins. We present different types of reporters and their experimental advantages and challenges, and describe how different strategies can interrogate exposure to antimicrobial host mechanism, pathogen response, inflicted damage, and impact on pathogen fitness at the single-cell level. We find many gaps in available tools but also exciting avenues to address these issues.

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Introduction

During infection, host receptors detect pathogens cells and activate antimicrobial effector mechanisms. These attacks can kill pathogen cells or inhibit their growth. However, many pathogens have evolved stress responses that prevent or repair host-induced damage and improve access to nutrients. The balance between host attacks and pathogen responses determines the ultimate outcome of the infection. Increasing evidence shows that some pathogen cells experience more intense host attacks than other pathogen cells elsewhere in the same tissue [1,2,3••,4–6]. Microbe-killing cells such as neutrophils can form segregated abscesses which may eradicate local pathogen cells, whereas pathogens cells in areas between abscesses survive. Inflammatory monocytes with high inducible nitric oxide synthase activity can also accumulate in certain tissue regions in which pathogen cells are exposed to toxic nitric oxide levels, while pathogen cells in other regions experience little nitric oxide. Multiple immune-cell types can also form highly organized structures such as granuloma in which pathogen cells experience special conditions. These heterogeneous host–pathogen encounters can have divergent individual outcomes which only together determine overall disease progression. This complexity provides challenges for experimental analysis. It also provides opportunities to determine underlying molecular mechanisms and their consequences for host and pathogen, as we can directly compare subsets of pathogen cells that are exposed or not to a particular host effector-mechanism within the same tissue at the same time. Even more importantly, understanding the factors that lead to pathogen eradication in some places but pathogen survival and even replication in other places might open completely new avenues for improved pathogen control and therapy of infectious diseases. To capitalize on these opportunities, it is essential to detect and characterize single pathogen cells that experience differential host attacks.

Single-cell analysis with fluorescent proteins

Single-cell analysis of mammalian cells has been revolutionized by single-cell RNA-sequencing (scRNA-seq) methods. These methods offer genome-scale transcriptional activities of individual cells in a high throughput manner. Methods for scRNA-seq of microbes are emerging [7•] but still have limited coverage and sensitivity. As an alternative, fluorescent protein reporters offer high specificity, sensitivity and flexibility for single-cell analysis by microscopy and flow cytometry (Table 1), but provide information for only one or at most a few different parameters per cell. In some cases, flow cytometry can purify many individual cells with specific fluorescence properties, providing sufficient material for comprehensive transcriptomics/proteomics of this particular pathogen subset.

Fluorescent proteins can be used to monitor changing conditions that alter their fluorescent properties within fractions of seconds to hours (e.g., changing absorption spectra at different pH values, changes in Förster resonance energy transfer — FRET upon ligand binding, temporal changes in TIMER proteins, etc.). Fluorescent proteins might change their localization within a cell over seconds to minutes (e.g., aggregation at DNA breaks). Fluorescent proteins can report changing promoter activities within minutes to hours when expressed from

Table 1

Fluorescent-based reporters for pathogen responses

Reporters	Features	Application	References
Fluorescent biosensors			
pHluorin	GFP Ratiometric	pH	[14]
pH-Lemon	CFP/YFP FRET-based Ratiometric	pH	[16]
FluBpH	EcFbFP/YFP FRET-based Ratiometric Stability from pH 3.4 to 10.8	pH	[15]
HyPer	cpYFP Ratiometric/pH-sensitive	H_2O_2	[17,18]
roGFP	GFP Ratiometric	Redox state (-SH/-SS-)	[35]
Frex	cpYFP Ratiometric/pH-sensitive	Redox state (NADH)	[19•]
iNAP	cpYFP Ratiometric/pH-resistant	Redox state (NADPH)	[19•]
PROPS	Green-absorbing proteorhodopsin Color changing over protonation	Membrane potential	[38]
Fluorescence Dilution	Division bisects fluorescence Best for few initial divisions	Number of divisions	[40,41]
TIMER	Color changing over time Oxygen-sensitive	Replication rate	[42,52••]
Green mKikumeGR	Photoconvertible by violet light Red color (photoconverted) Green color (non-photoconverted)	Replication rate	[43•]
ATeam1.03^{YEMK}	CFP/YFP FRET-based Ratiometric Medium affinity for ATP	Relative ATP levels	[53]
iATPSnFRs	cpSFGFP Intensiometric	ATP levels	[54]
mRuby-iATPSnFR$^{1.0}$	mRuby/cpSFGFP Ratiometric	ATP levels	[54]
GEPIIs	CFP/YFP FRET-based Ratiometric Temperature-dependent	K^+ levels	[55]
Stress-responsive promoters			
P_{katG} /P_{ahpC}	OxyR regulon	H_2O_2	[20]
P_{hmpA}	NsrR regulon	NO	[22,23,24]
$P_{hspX'}$	*dos* regulon	NO/hypoxia	[32]
P_{iroB}	FUR regulon	Iron starvation	[28]
P_{recA}	LexA regulon/SOS response	DNA damage	[34]
P_{micA}	Sigma E regulon	Envelope integrity	[36]
P_{rRNA}	Ribosomal promoter	Ribosomal activity	[48]
Translational fusions			
RecA-GFP	DNA repair/SOS response	DNA damage	[33]
SSB-GFP	Replisome component	Replication status	[49]
ParB-GFP/ParB-mCherry	Origin and terminus replication regions	Replication status	[50]

transcriptional fusions. Most fluorescent proteins are stable over hours to days resulting in very slow responses to declining gene expression, but destabilized variants can report current expression levels more accurately. Alternatives to fluorescent proteins for transcriptional studies might include fluorogenic RNA-based sensors that report transcription without delays due to translation, folding, and fluorophore maturation [8].

Rapid changes in fluorescent-protein properties are suitable for direct monitoring with live-cell imaging of cell-culture infection models or intravital imaging of *in-vivo* models. By contrast, these readouts are likely to change during tissue preparation/fixation thus becoming less informative about the *in-vivo* situation. The more slowly changing transcriptional reporters are often more suitable for *ex-vivo* analysis.

Most fluorescent proteins have weak or no toxicity for most organisms. However, fluorescent proteins such as GFP and tagRFP can generate reactive oxygen species such as superoxide and hydrogen peroxide when exposed to NADH or NADPH [9]. Furthermore, fluorescent proteins can leak from live bacterial cells [10] suggesting

some membrane perturbation. In addition, the extra metabolic burden of producing a protein that is useless for the pathogen can compromise pathogen fitness [11]. Fluorescent protein levels should thus be as low as possible for sufficient signal-to-background ratios. Green–yellow fluorescent proteins might need higher expression levels than red fluorescent proteins because of strong flavin autofluorescence in inflamed host tissues. However, adapted narrow bandpass filters (e.g., a bandpass filter transmissible for light with 500−520 nm for GFP, instead of common FITC filters with transmission 510−550 nm) can partially mitigate this background problem. Fluorescent proteins with emissions in the infrared range have low tissue background and superior tissue penetration but still limited brightness. In addition to signal-to-background, the dynamic range of the reporter (induced versus uninduced) is key for interpretable results. Ratiometric reporters (readout is a ratio between fluorescence intensities measured at two different wavelengths) reduce the impact of variable fluorescent protein concentration thereby enabling detection of even subtle signal changes. For transcriptional reporters that are based on a single fluorescence intensity (intensiometric), our experience suggests that the dynamic range should be tenfold or more to be able to detect signals against stochastic fluctuations in fluorescent protein content, translational efficiency, autofluorescence of the pathogen cell, and differences in cell size.

Finally, the requirement of oxygen for fluorophore formation in most fluorescent proteins imposes limitations in host tissues with low oxygen supply. For GFP, incomplete fluorophore formation *in vivo* can be partially rescued by a 15 min *ex-vivo* incubation in fully oxygenated buffers. Oxygen-independent fluorescent proteins provide interesting alternatives [12]. Flavin-binding proteins overlap strongly with tissue autofluorescence and have low extinction coefficients limiting their brightness although recent variants look promising [13]. Bilirubin-binding proteins have attractive spectral properties but require a heterologous bilirubin biosynthesis pathway draining intermediates from heme metabolism with yet unclear fitness effects. However, once bilirubin delivery is optimized for minimal interference, these proteins might enable unique opportunities for *in-vivo* detection.

Detecting antimicrobial mediators

Fluorescent proteins enable direct detection of some antimicrobial host molecules. Specifically, pathogens experience acidic conditions when residing extracellularly in some inflamed regions, or after phagocytosis by certain host cell-types. Some fluorescent proteins respond to acidic pH by changing their spectral properties with suitable pK values. The YPF variant phLuorin is widely used as a pH sensor but seems to have some limitations [14]. Newer pH sensors might offer advantages [15,16]. pH sensors are commonly expressed in the cytosol of pathogens and their readouts thus reflect the pathogen's capability to compensate for external pH changes. Targeting the reporters to the pathogen surface (or the periplasm of Gram-negative bacteria) would more faithfully report acidic host attacks. On the other hand, pH measurements in the cytosol indicate conditions in the most sensitive pathogen compartment.

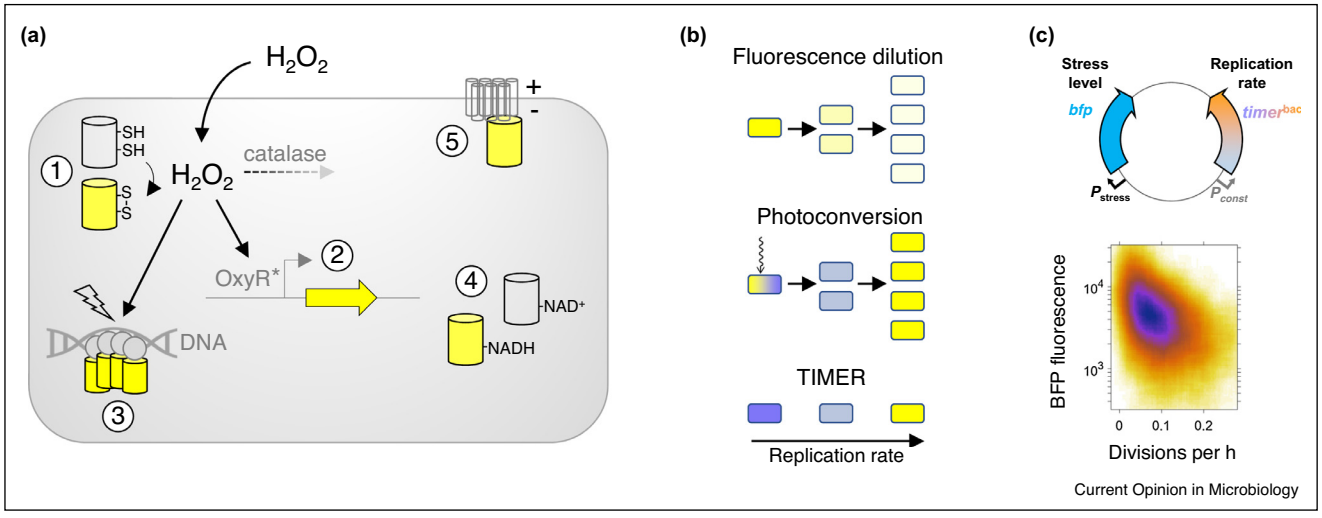
Another common antimicrobial effector mechanism employs reactive oxygen species (ROS). Fluorescent proteins of the HyPer family [17] react directly with hydrogen peroxide and report such attacks in real-time [18] (Figure 1a). Again, cytosolic expression leads to reporting of ROS levels as they reach the primary location of sensitive targets in the pathogen cytosol. This type of sensor does not react directly with other relevant ROS such as superoxide and hypochlorite but would detect superoxide via its spontaneous disproportionation reaction product peroxide. The pH-dependency of HyPer signals requires careful controls [17]. Other pH-independent sensors might offer advantages over HyPer [19•].

Antimicrobial mediators can also be detected based on their reaction with transcriptional regulators. As an example, hydrogen peroxide oxidizes two cysteines in the oxidative stress regulator OxyR. The resulting disulfide bridge leads to a conformational change resulting in stimulatory interactions with RNA polymerase to activate expression of target genes [20] (Figure 1a). Monitoring of transcriptional fusions of target promoters (such as P_{katG} or P_{ahpC}) to fluorescent proteins can provide an indirect readout for *in-vivo* hydrogen-peroxide exposure of *Salmonella* cells [21,22] (Figure 2a). As another example, reaction of nitric oxide with the iron–sulfur cluster of the nitrite-sensitive repressor NsrR leads to detachment of the repressor from the DNA and de-repression of corresponding target promoters [23]. Transcriptional fusions to one such promoter (P_{hmpA}) enable visualization of *in-vivo* nitric-oxide exposure of *Salmonella* and *Yersinia* cells [22,24].

In addition to direct attack with toxic molecules, hosts can also restrict pathogen growth by nutrient deprivation ('nutritional immunity' [25]), while pathogens might trigger increased nutrient supply ('nutritional virulence' [26]). As an example for detecting nutritional immunity, binding of ferrous iron to the ferric uptake regulator FUR increases its binding to DNA resulting in repression or activation of target promoters [27]. A reporter fusion to one such promoter (P_{iroB}) detects *in-vivo* iron starvation in *Salmonella* cells [28].

It is important to note that such transcriptional reporters might respond to multiple different stimuli because (i) the pathogen itself might produce similar molecules as

Figure 1



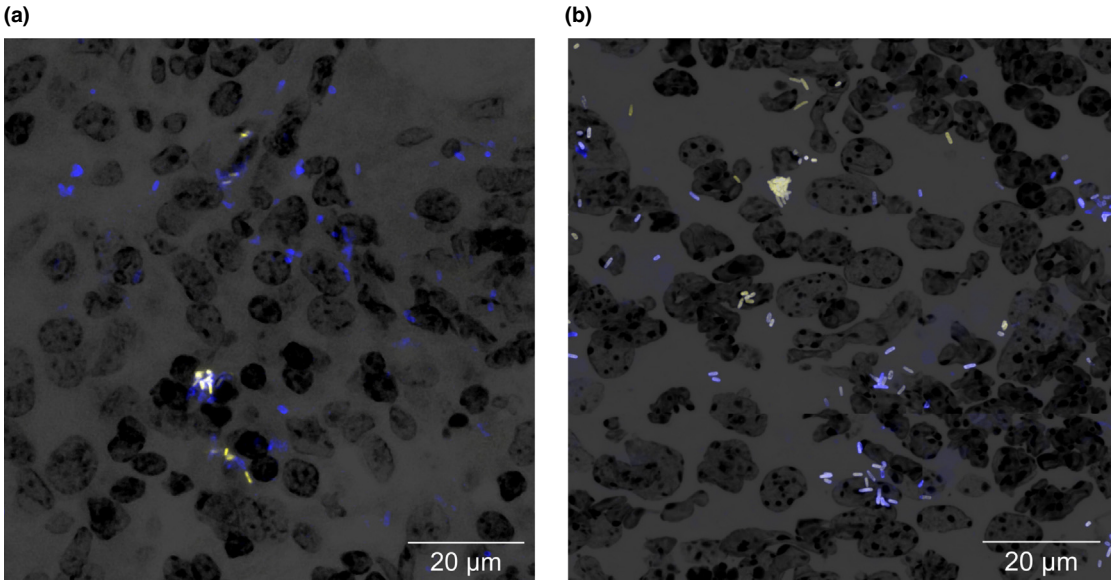
Interrogation of pathogen responses to host immunity at the single-cell level.
(a) Strategies for reporting exposure to antimicrobial mediators and its consequences using hydrogen peroxide as an example. Detection can focus on the antimicrobial molecule itself (1), transcriptional responses activated by exposure (2), inflicted damage (3), or consequences for bacterial physiology including metabolite concentrations (4) and membrane potential (5).
(b) Strategies to follow pathogen replication. In fluorescence dilution, cells are pre-loaded with fluorescent protein. Distribution of this protein to daughter cells leads to diminishing fluorescence intensities. In photoconversion, intense laser light switches fluorescence colors. Proteins with switched color are diluted with each division and replaced by new unswitched fluorescent protein leading to a gradual color change. The TIMER protein changes color with growth rate because a slowly maturing long-wavelength form can only reach detectable levels in cells with low replication rate.
(c) Strategy for correlating replication rate with stress levels in the same cell. Upper panel: schematic description of a dual reporter plasmid with stress-inducible *bfp* expression and constant $timer^{bac}$ expression. Lower panel: *in-vivo* flow cytometry data for *Salmonella* carrying a dual reporter plasmid with *bfp* under the control of the $P_{mgtCBRcigR}$ promoter/leader which is induced by magnesium starvation.

the host (e.g., *Salmonella* generate endogenous nitric oxide [29]), (ii) the sensor is responsive to various induces (e.g., OxyR can be activated both by hydrogen peroxide and nitric oxide, although the latter is questionable under physiological conditions [30]), and/or (iii) the target promoter integrates signals from different transcription factors (e.g., P_{hmpA} is regulated by NsrR, FUR, FNR, DksA, MarA, MetR as well as the small regulatory RNA SdsN [31]). Identifying host factors that trigger reporter responses thus requires additional evidence: (A) Perturbing candidate host mechanisms pharmacologically or genetically can clarify their role in reporter induction. As an example, *Salmonella* subsets show detectable activity of P_{hmpA}-*gfp* during infection of wildtype mice but not in mice deficient for inducible nitric oxide synthase (iNOS) suggesting that host-generated nitric oxide triggered this *Salmonella* response [22]. However, perturbing central host defense mechanisms might have secondary effects that could complicate interpretations. Moreover, suitable tools for such perturbations are not available for all candidate mechanisms. (B) Purification and comprehensive analysis of responding pathogen subsets might reveal additional pathogen responses that are compatible with a certain house attack mechanism. As an example, the *Salmonella* subset with high P_{hmpA} activity contains increased levels of additional proteins with a direct functional link to nitric oxide exposure, but no evidence of differential iron starvation or oxygenation [22]. (C) Comparison of different reporter fusions with potentially overlapping induction mechanisms can disentangle different stimuli. As an example, *Salmonella* cells carrying dual reporters for P_{hmpA} and P_{katG} show uncorrelated responses suggesting that P_{katG}-stimulating OxyR is mainly activated *in vivo* by hydrogen peroxide with little impact of nitric oxide [22]. Finally, promiscuous responses of reporters can provide information about global stress levels without determining the exact induction mechanism. As an example, P_{hspX} fusions are used to monitor exposure of *Mycobacterium tuberculosis* to nitric oxide and/or hypoxia [32].

Detecting damage

Pathogen cells can mitigate antimicrobial host attacks with permeability barriers and/or detoxification mechanisms. Only if such defense mechanisms are overwhelmed, cellular damage and potential fitness defects might occur. Fluorescent proteins can help to detect such damage but applications in host–pathogen studies

Figure 2



Examples of heterogeneous *Salmonella* properties in infected mouse spleen as detected by confocal microscopy of cryosections.
(a) *Salmonella* expressing mCherry from a constitutive promoter (shown in false-color blue) and GFP from the P_{katG} promoter, which responds to host-generated hydrogen peroxide (shown in false-color yellow). Most bacteria have undetectable P_{katG} activity, but some cells have strong responses suggesting inhomogeneous exposure to reactive oxygen species. Host nuclei stained with DAPI are shown as an inverted grey image.
(b) *Salmonella* expressing the replication reporter TIMER^{bac} that changes fluorescence color from orange (shown as blue) to green (shown as yellow). Bacteria have various shades of blue to yellow indicating heterogeneous replication rates. Host nuclei stained with DAPI are shown as an inverted grey image.

are still rare. As an example of studies in axenic bacterial cultures, fusions of fluorescent proteins to the DNA recombination/repair protein RecA rapidly localize to damaged DNA where they form fluorescent spots [33] (Figure 1a). These RecA filaments promote autocatalytic cleavage of the transcriptional repressor LexA which initiates the transcriptional response to DNA damage (SOS response). As part of this response, the P_{recA} promoter gets de-repressed which can be detected using transcriptional fusions [34]. As another example, oxidative damage and perturbed respiration can be monitored using various fluorescent proteins that respond to NADH, NADPH, the redox state of glutathione, or peroxide levels [19•,35] (Figure 1a). As a third example, misfolded proteins in the periplasm activate the extracytoplasmic function Sigma factor E. Sigma E stimulates expression of multiple target genes including the small non-coding RNA MicA which can be exploited with transcriptional fusions [36]. Finally, monitoring of membrane potential with fluorescent proteins is an active field in neurobiology [37•] but might also offer fascinating insights into bacterial membrane potential [38] including during host attacks with antimicrobial peptides (Figure 1a).

Detecting consequences of antimicrobial attacks

Pathogens might repair damage or use compensatory mechanisms that preserve fitness. Only when these protective mechanisms are insufficient, pathogens will die or suffer growth retardation. Pathogen death in host environments is often associated with compromised cell-envelope integrity leading to loss of cytoplasmic fluorescent proteins. This can be followed by live imaging. Alternatively, detection of more stable remnants of pathogen corpses with no associated fluorescent protein signal reveal killed pathogen cells. As an example, an antibody to lipopolysaccharide can detect killed *Salmonella* cells that have lost their fluorescent protein content [39].

Pathogen replication rates have been of particular interest because of their impact on antibiotic clearance. Pathogen replication can be followed directly by live-imaging of cell-culture infections or intravital microscopy of infected host tissues. Larger single-cell data sets can be obtained with methods that provide replication rates even with snapshot measurements (Figure 1b). The total number of divisions after start of infection can be determined based on the distribution of a pre-formed fluorescent protein to

daughter cells which thereby become dimmer and dimmer (fluorescence dilution) [40,41]. This method works best for the first few divisions because of declining signal-to-background. It provides the entire number of divisions since start of infection instead of current rates and thus cannot resolve fluctuations in replication rate. The initial high load of fluorescent protein might cause a fitness disadvantage. Alternatively, pathogen replication rate can be determined using fluorescent proteins that change color over time (so-called TIMER proteins) [42]. In non-dividing cells, TIMER has sufficient time to fully mature, while rapid replication dilutes the TIMER protein before the late color emerges resulting in dominance of the early color (Figure 2b). TIMER maturation depends on oxygen concentration limiting this method to conditions/tissues with homogeneous oxygen supply. A third technique relies on turnover of a photo-switchable fluorescent protein after photoconversion which correlates with replication [43•]. An exciting fourth option could be combination of heavy water (^2H_2O) labeling in infected hosts [44,45] with Raman microscopy and cell-sorting [46•] or mass spectroscopy [47]. As a fifth option, transcriptional fusions of ribosomal promoters vary with replication rate [48] but might also respond to unrelated stimuli. Finally, fusions of fluorescent proteins to factors associated with cell cycle such as the single-stranded DNA binding protein SSB [49], FtsZ, or the chromosomal origin of replication (*oriC*) and the replication terminus region (*terC*) [50], can highlight the fraction of actively replicating pathogen cells, but do not provide single-cell replication rates.

Pathogen replication and survival in host environments is clearly heterogeneous but underlying molecular mechanisms are rarely understood [51], because tools enabling simultaneous monitoring of replication rates and stress levels/molecular alterations in the same single cells are largely lacking. To approach this issue, we recently combined the replication-rate reporter TIMER^{bac} with a non-overlapping fluorescent protein (mTagBFP2) expressed from stress-responsive promoter fusions (Figure 1c). Using this method, we could show that magnesium but not zinc starvation is a key contributor to growth heterogeneity of *Salmonella* in mice with functional SLC11A1 [52••]. Alternative methods could determine how rapidly replicating pathogen cells differ from cells with lower fitness in terms of metabolite concentrations, enzymatic activities, energy levels [53,54], ion gradients [55], etc. Fascinating approaches for similar questions are currently being developed for microbial communities [56]. Some of them might be applicable to host–pathogen interactions.

Conclusion

Single-cell approaches have revealed extensive heterogeneity in host–pathogen interactions. However, the underlying molecular mechanisms and the relevance for disease progression are still poorly understood. Additional tools are required to study microbial physiology in host contexts at the single-cell level. In particular, we lack a versatile toolbox to interrogate pathways that compromise pathogen fitness. However, emerging methods that are primarily being developed for mammalian cells or microbial communities open promising avenues to address these issues in infectious diseases.

Conflict of interest statement

Nothing declared.

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