Applications of microscopy in *Salmonella* Research

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Abstract:

Salmonella enterica is a Gram-negative enteropathogen that can cause localised infections, typically resulting in gastroenteritis, or systemic infection e.g. typhoid fever, in humans and many other animals. Understanding the mechanisms by which *Salmonella* induce disease has been the focus of intensive research. This has revealed that *Salmonella* invasion requires dynamic cross-talk between the microbe and host cells, in which bacterial adherence rapidly leads to a complex sequence of cellular responses initiated by proteins translocated into the host cell by a type 3 secretion system. Once these *Salmonella*-induced responses have resulted in bacterial invasion, proteins translocated by a second type 3 secretion system initiate further modulation of cellular activities to enable survival and replication of the invading pathogen. Elucidation of the complex and highly dynamic pathogen-host interactions ultimately requires analysis at the level of single cells and single infection events. To achieve this goal, researchers have applied a diverse range of microscopy techniques to analyse *Salmonella* infection in models ranging from whole animal to isolated cells and simple eukaryotic organisms. For example, electron microscopy and high resolution light microscopy techniques such as confocal microscopy can reveal the precise location of *Salmonella* and its relationship to cellular components. Widefield light microscopy is a simpler approach with which to study the interaction of bacteria with host cells and often has advantages for live cell imaging, enabling detailed analysis of the dynamics of infection and cellular responses. Here we review the use of imaging techniques in *Salmonella* research and compare the capabilities of different classes of microscope to address specific types of research question. We also provide protocols and notes on some microscopy techniques used routinely in our own research.

Keywords:

Salmonella, infection, imaging, microscope, widefield microscopy, confocal laser scanning microscopy, fluorescent staining, live cell imaging, scanning electron microscopy

1. Introduction

1.1 *Salmonella* infection

Salmonella is a Gram-negative, flagellated enteropathogen which can cause self-limiting gasteroenteritis or in some cases more severe systemic infections e.g. typhoid fever and secondary bacteremia. Despite improvements in food safety, *Salmonella* continues to be both a social and economic burden responsible for significant morbidity and mortality worldwide. Non-typhoidal *Salmonella* (NTS) is estimated to cause 93.8 million serious infections worldwide per year and 155,000 deaths (1) whilst the exclusively human adapted serovars *S.* Typhi and *S.* Paratyphi are estimated to cause more than 27 million cases of typhoid fever, of which over 200,000 are fatal (2). The virulence of *Salmonella enterica* depends on its ability to enter and survive in host cells. Primary infection occurs in the gut after ingestion of contaminated food or drink. The bacterium travels through the digestive system, surviving the low pH conditions of the stomach, to enter the small intestine. Environmental conditions within the lumen of the small intestine activate *Salmonella* virulence gene expression, promoting cellular invasion. A type 3 secretion system (T3SS) encoded by *Salmonella* pathogenicity island 1 (SPI-1) triggers cellular responses including extensive actin cytoskeleton rearrangement, producing “membrane ruffles” on the surface of the epithelial cell (3,4) which facilitate bacterial uptake.

Salmonella invade preferentially, but not exclusively, via non-phagocytic M cells present in the epithelium overlaying the gut-associated lymphoid tissue (5-7). The bacterium exploits the antigen uptake and transport function of M cells as a portal for entry into epithelial cells and subsequent dissemination into other cell types, including macrophages. After initial invasion of host cells, bacteria survive and replicate within a specialised membrane-bound compartment called the *Salmonella*-containing vacuole (SCV). From within this vacuolar niche, and with the aid of a second T3SS encoded by SPI-2, *Salmonella* are able to proliferate and avoid degradation by manipulating membrane trafficking, leading to a divergence from the classical endosome/lysosome pathway.

Understanding the mechanisms underlying *Salmonella*-host cell interactions advances our knowledge in both microbial pathogenesis and cell biology. Use of microscopy in this area has provided researchers with a powerful tool to study the intimate and complex cross-talk between bacteria and host cells at a single cell, and single event, level (8-20). Live cell and fixed cell microscopy has provided a robust tool for revealing the dynamics of *Salmonella* adherence, membrane ruffling (8-13), SCV biogenesis (8,9,14-16) and bacterial replication within infected cells (17-19). Advances in microscopy, together with the development of fluorescent protein-based reporters has helped delineate signalling and trafficking events in infected cells (8,13-16,19,20). Similarly, GFP-based reporters and microscopy have advanced understanding of virulence gene expression and the extent of population heterogeneity (21, 22).

There is insufficient space to discuss in detail the intricate relationship between *Salmonella* and host cells but information on these topics can be found elsewhere in this volume and in other comprehensive reviews (4, 23, 24). Here we will focus on the ways microscopy is contributing to research in this field.

1.2 Applications of microscopy in *Salmonella* research

1.2.1 *Introduction to microscopy techniques*

Although we will mainly discuss the use of light microscopy techniques, including conventional phase contrast, fluorescence and confocal microscopy, it is worth emphasising that a much broader range of microscopy techniques has been used to study bacterial morphology and the processes involved in infection. For example, transmission electron microscopy (TEM) provides morphological information on *Salmonella* surface structures such as pilli, flagella and T3SS, even yielding molecular level structural information when high resolution TEM is coupled with advanced image processing and analysis (25-28). In conjunction with TEM, immunogold labelling offers a useful biological tool to localise proteins of interest and has been used to locate outer membrane proteins and confirm expression of the Vi capsular polysaccharide in *Salmonella* (29, 30). Scanning electron microscopy (SEM) is also an invaluable technique for revealing the detailed

surface structure of individual bacteria, biofilms and the changes induced on host cells during infection (5, 7, 11, 31-33). We will briefly discuss some examples of the use of EM and processing techniques we have employed to examine the morphology of *Salmonella* and of membrane ruffles induced during infection of epithelia (Fig 1) but refer the reader to other papers for detailed technical aspects of the application of EM to study aspects of bacterial infection (34, 35).

Atomic force microscopy (AFM) is also capable of providing high resolution images of surface structures including those on living bacteria (36, 37) and, although it has yet to be widely used to study *Salmonella* infection, it has been employed to study the morphology of protein secretion and translocation pores of *E. coli,* bacterial motility and biofilm formation (38-43).

Light microscopy techniques are more frequently used to study the interaction of bacteria with cells because they are more readily accessible and, though they fall short of some other techniques in terms of resolution, they are very flexible and can be used to study dynamic processes occurring in living cells. We will discuss some technical aspects of these methods later but will first give a brief introduction to the types of imaging techniques available and how they have been applied to study *Salmonella* infection. Although we are concentrating on the most widely used and accessible techniques it is likely that other techniques will start to become more commonplace in *Salmonella* research. For example, multiphoton (MP) microscopy utilises the greater penetration of longer wavelength light to study fluorescent entities deep within complex environments and therefore has potential applications in studying the dynamics of host-pathogen interactions during infection of intact tissues as well as intricate biofilm structure (44-46). This approach was recently used to study *Salmonella*-induced recruitment of dendritic cells into the intestinal epithelium of living mice (47). Within the past few years, a number of optical and statistical methods have been developed to overcome the traditional diffraction limit to optical resolution improving resolution of LM from 200-250nm to <100nm (reviewed in 48, 49). These ‘super-resolution’ microscopy techniques are an area of rapid technological development that will offer new opportunities to bridge the gap between convention LM and EM resolution. Although we are unaware of any publications reporting application of super-resolution microscopy to image

Salmonella, the recent application of PALM and structured illumination microscopy to image in unprecedented detail the DNA repair machinery, cell wall architecture and cytokinesis mechanisms in bacteria (50-52) provides proof of principle for their potential application in *Salmonella* research.

Many researchers use a combination of microscopy techniques to address specific questions, reflecting the fact that each has distinct advantages for specific purposes. Having said that, most researchers will not have the luxury of access to a vast range of microscopy systems and here we intend to concentrate primarily on those techniques that are most widely available, namely widefield microscopy (WFM) and confocal laser scanning microscopy (CLSM).

It is necessary to understand some basic principles underlying LM techniques in order to assess their relative merits for certain applications. The reader requiring more detailed information is referred to other papers dealing with these issues in greater depth (53-55). WFM can also be referred to as conventional light microscopy and in its simplest form involves a standard upright or inverted microscope (often with fluorescence capabilities) to which a camera (e.g. CCD camera) is added to enable simple image acquisition. The term ‘widefield’ refers to the fact that light is detected from a broad focal depth so the resulting image approximates that seen by the user through the microscope eyepieces, including both ‘in-focus’ and ‘out-of-focus’ information. WFM imaging systems are highly adaptable, for example they may incorporate shuttering, focus drives and filter changers to enable the user to automate rapid switching between imaging parameters. These capabilities, together with the high sensitivity of camera systems, which allows rapid image acquisition and minimisation of light exposure, makes WFM a popular choice for live cell imaging. Cameras and illumination systems are areas of rapid technological development leading to increased sensitivity, speed and stability of WFM systems in recent years. This progress seems likely to continue and offers new opportunities for the application of WFM in live cell imaging by optimising acquisition of quantifiable image data while minimising the risk of imaging conditions interfering with the cellular processes being investigated.

Since its commercialisation in the 1980’s, confocal microscopy has become a relatively routine research tool, with CLSM systems in particular being widely available. In CLSM, an image

of fluorescence (or reflectance) is acquired point-by-point, or more rarely line-by-line, as a laser is scanned across a field of view. Light emanating from the sample follows a reverse path (is 'descanned') and passes through an aperture (pinhole) to a detector, typically a photomultiplier tube (PMT). In allowing light from only one plane of focus to reach the detector, the confocal aperture facilitates 'optical sectioning', enhancing axial resolution and giving CLSM its major advantage over WFM. Most CLSMs allow simultaneous detection of different fluorophores by selectively directing different wavelengths of emitted light to different detectors. The ability to rapidly switch between excitation wavelengths using an acousto-optic tuneable filter (AOTF) to sequentially excite different fluorophores simplifies separation of their signals and is a standard feature of many modern CLSMs.

When considering CLSM and WFM we are discussing systems with distinct fluorescence imaging capabilities; CLSM providing the best axial resolution achievable with commonly available systems but generally at the cost of sensitivity, speed, increased risk of photodamage, and price. An important caveat to the above generalisation is that recent advances in detector and scanning technology have increased the sensitivity and speed of CLSM systems, although these advances are yet to become standard features. It is also worth mentioning an additional type of 'confocal' microscopy technique, that of spinning (or Nipkow) disk systems, which in some respects occupy a position in between CLSM and WFM. These systems illuminate specimens with laser light spread across a field of view via an array of pinholes which limit detection to a single level of focus and use a camera to simultaneously detect emitted light from the entire field. Although faster and less prone to inducing photodamage than conventional CLSMs, which gives them advantages for live cell imaging, spinning disk systems are less flexible in terms of magnification and depth of focus, and provide lower axial resolution than point-scanning confocals. Spinning disk systems offer a very good alternative for imaging at improved axial resolution compared with WFM and have been used extensively for live cell imaging e.g of *Salmonella* infection of cultured cells (18, 19) and also for imaging *Salmonella* biofilms (56).

The other principle advantage that CLSM has over WFM and spinning disk systems is that its use of point-scanning allows flexibility over magnification (by 'zooming' on a sub-region of the specimen). Control of scan geometry also makes CLSM inherently applicable to techniques that require selective illumination of defined areas (photo-bleaching, photo-activation, uncaging). Selective photobleaching can, for example, be used to study mobility of GFP-tagged cellular proteins in the FRAP (fluorescence recovery after photobleaching) technique which has found widespread application in cell biology. For example, FRAP has been used to track the diffusion of effector proteins along *Salmonella*-induced filaments during infection (57). While addition of photo-bleaching lasers to WFM and spinning disk confocals can also enable such techniques on these systems and do this in a way that can be more interactive than CLSM systems, the flexibility of point-scanning systems usually gives them an advantage in this respect.

Up until now we have emphasised increased axial resolution as a major advantage of CLSM but it should always be remembered that this is accompanied by a decrease in sensitivity due to the large proportion of photons that are discarded at the confocal aperture. Moreover, optimal resolution can be a disadvantage where simultaneous detection of fluorescence in a broad depth of field will accelerate data acquisition. Typically, CLSM systems allow the user to open the confocal aperture to increase sensitivity and this is an option well worth exploring whenever sensitivity is favoured above optimised resolution, as is usually the case in live cell imaging.

In emphasising the low axial resolution of WFM systems we have yet to consider improvements that can be made subsequent to image acquisition by image processing. The application of deconvolution algorithms to stacks of WFM images allows reassignment of 'out-of-focus' light to its point of origin and can result in marked improvement of image clarity (Fig 2). WFM coupled with deconvolution can often perform at least as well as CLSM in resolving fluorescent structures, especially when these are of relatively low intensity and samples are relatively thin (58). However, deconvolution is most effective when stacks of images at relatively narrow focus increments are acquired in a way that optimises sampling frequency (Nyquist sampling) (54,58, 59). Since deconvolution relies on accurate imaging of both in-focus and out-of-focus signal within

multiple images, it is reliant on the stability of the imaging systems and susceptible to any movement of fluorescent entities during stack acquisition. The effectiveness of deconvolution depends on the robustness of the algorithms applied and these vary significantly between commercial software packages. Because deconvolution can be problematic for reasons outlined above, high resolution images can generally be obtained more simply using CLSM and this technique is thus preferred by most researchers unless the advantages of WFM outweigh these issues e.g. when fluorescent signal is necessarily low. It should also be noted that deconvolution can also enhance confocal data by removing remnant out-of-focus information and signal noise that arises from inherent low signal-to-noise ratio of CLSMs (Fig 2). It is generally accepted that CLSM is likely to out-perform WFM/deconvolution when thicker specimens with more complex distributions of fluorescence are to be imaged, since the contribution of 'out-of-focus' fluorescence has a more profound effect on image resolution in such circumstances (54, 58). For this reason CLSM has distinct advantages when studying *Salmonella* infection of polarised epithelia and intact tissues.

1.2.2 *Microscopical localisation of Salmonella in cells and tissues*

Monitoring bacterial invasion and precise localisation of *Salmonella* within cells and tissues is a routine requirement in *Salmonella* research. *Salmonella* invasion of epithelial cells in guinea pig ileum was first observed in TEM studies (60) and subsequent studies built on these observations using a combination of light and electron microscopy (SEM and TEM) techniques (5, 6, 61). Examination of tissues by TEM alone is relatively laborious and for this reason most studies will examine a limited number of cells and thus potentially bias observations and overlook significant events (62). Therefore, where the optimal resolution of TEM is not required, methods that allow *en face* imaging of extensive areas of epithelium have a distinct advantage in facilitating observation of interactions of *Salmonella* with many cells. For example, CLSM has been used to localise *Salmonella* adhered to and within M cells in intact Peyer's patch tissue preparations, while parallel studies with SEM - sometimes examining the same cells previously imaged by CLSM in a primitive

form of CLEM (correlative light electron microscopy), allowed surface morphology of *Salmonella*-infected epithelial cells to be examined (5, 62, 63). Similar techniques have also been applied to precisely localise infrequently encountered *Salmonella* within thick sections of intestine and liver (64, 65).

Identification of *Salmonella* using specific antibodies in conjunction with fluorescently labelled secondary antibodies rapidly became the main method for localising *Salmonella* in relation to cellular components or in specific cell types (66, 67). Antibody staining has also been used to differentiate between, and quantify, internalised and external bacteria after infection of cells, exploiting the fact that bacteria within intact eukaryotic cells are inaccessible to externally applied antibodies unless the plasma membrane is permeabilised. This method is sometimes regarded as an alternative to the more commonly used, and arguably less laborious, gentamicin-protection assay of bacterial internalisation. However, differential immunolabelling of adhered and invaded bacteria has distinct advantages in that adherence and invasion are quantified in the same cells, it provides information on heterogeneity in distribution of *Salmonella* within cells and it allows simultaneous monitoring of cell damage - cytotoxicity and cell loss being a potential source of serious artefacts in the gentamicin-protection assay. Variations of differential immunolabelling technique have been used. One uses transfer of cells to ice-cold PBS before application of ice-cold antibodies to label external *Salmonella* prior to fixation, and permeabilisation with methanol to allow access of antibodies to internalised bacteria in a second round of labelling with a different fluorophore (68, 69). We have adopted a variation of this technique that allows more flexibility with the timing of labelling by using paraformaldehyde (PFA) to fix, but not permeabilise, cells (22). The samples can then be labelled with antibodies to localise external bacteria, permeabilised with Triton X-100, then re-stained with antibodies and an alternative fluorophore to label all bacteria (described in section 3.3.2). In our hands, each of these immunolabelling methods has proved more reproducible for assaying bacterial invasion than the gentamicin-protection assay. Immunolabelling to discriminate external and internal bacteria can also be employed along with additional antibodies, or GFP expression, to localise cellular components or transfected cells,

enabling quantification of invasion in transfected versus non-transfected cells to investigate the effect on infection of modulating host protein expression (70).

Expression of GFP or alternative fluorescent proteins such as mCherry is now widely used to localise *Salmonella* since it avoids the need for additional labelling steps and enables live cell imaging (18, 19, 71, 72). Genetic manipulation of bacteria, although arguably more time consuming and technically challenging to engineer, can provide users with real time information which can be applied *in vivo*. Another advantage of this technique is that it provides less complicated sample preparation compared to antibody labelling techniques. GFP labelling has also been used in conjunction with immunolabelling to differentiate internalised bacteria from external ones using a simplified technique based on that described later (section 3.3.2), only requiring fixation and one round of antibody labelling to localise external bacteria, GFP marking both external and internal bacteria (68). Alternative fluorescent proteins, such as GFP and mCherry, can discriminate between bacterial populations (20) and offer the potential to simultaneously follow infection of distinct *Salmonella* strains during co-infection. It is important to consider the impact that high levels of fluorescent protein expression might have on bacterial fitness and to perform appropriate controls to test for this possibility. Indeed some studies have highlighted adverse effects of plasmid carriage on *Salmonella* infection (72, 73). *S*ingle-copy expression of GFP is less likely to affect bacterial behaviour (21).

In addition to providing a method of detecting and enumerating *Salmonella*, GFP expression may be coupled to specific promoters to monitor expression of particular genes or sets of genes, either microscopically or through the use of flow cytometry or fluorescence-activated cell sorting (FACS). The ‘differential fluorescence induction’ technique of Valdivia and Falkow (74), which utilises GFP and FACS, identifies promoters whose expression depends on particular environmental stimuli, such as low pH, or are expressed during infection of specific cells or tissues (74, 75). GFP-promoter constructs have been shown to report gene induction in *Salmonella* as accurately as *lacZ* gene fusions, and are being used successfully to monitor gene induction *in vitro*

and during infection of mammalian cells (18, 21, 22) and have the potential to be applied in animal infection models.

Fluorescence microscopy, including CLSM, has also been used to locate translocated effector proteins within infected cells using antibodies to the effector protein itself or, more commonly, epitope tags (76-78). Use of full length GFP as a means of fluorescently labelling effectors has not been possible since the GFP molecule blocks transfer into host cells. Other methods have recently been devised to measure the rate of translocation of effector proteins that, due in part to the rather small numbers of molecules translocated into host cells, have proved difficult to localise by immunolabelling. For example, *Salmonella* effector translocation has been studied using a sensitive method employing tagging of effector proteins with TEM-1 beta-lactamase. Treatment of cells with a fluorescent lactamase substrate CCF2/AM that is sequestered in the cytoplasm allows detection of TEM-1-tagged proteins translocated into the cells (79). By effectively amplifying the signal from individual effector molecules this method enhances detection sensitivity and, despite its inability to precisely localise effector proteins (due to diffusion of fluorescent enzyme product) has proven potential as a sensitive single cell assay of translocation. Quantitative immunolabelling of SipA within *Salmonella* after defined periods of interaction with host cells (determined by live cell imaging prior to fixation and labelling) has been used to estimate the rate of transfer of SipA into the host cell (10).

Detection of SopE2 and SptP delivery into host cells has also been examined using the tetracsyteine-dependent FlAsH tag to fluorescently label effector protein within bacteria and monitor its secretion as loss of signal during infection of host cells (80). An elegant ‘split GFP’ method was also employed to detect effector delivery, whereby effectors were linked to a small part of GFP, to enable translocation. Reconstitution of full GFP was then enabled by expression of the remaining part of the molecule in host cell cytoplasm so that effector delivery could be effectively monitored by appearance of GFP fluorescence (57).

1.2.3 *Imaging cellular responses to Salmonella infection*

When microscopy methods were first used to study *Salmonella*-infected cells and tissues, it became clear that entry of *Salmonella* into epithelial cells involved major changes in cell morphology. Formation of the characteristic *Salmonella*-induced 'membrane ruffles' was studied extensively using TEM (60, 61, 81) and SEM (61, 62, 69, 82), and fluorescence labelling with LM helped determine how the redistribution of actin and other cytoskeletal proteins promoted formation of these ruffles (3, 61, 67). With the discovery of the SPI-1 T3SS, attention has turned to unravelling the roles of the various effector proteins in triggering cellular responses associated with bacterial pathogenesis.

Much of the research studying the mechanisms of *Salmonella* invasion has been based on infection of cultured cells. Such model systems have clear advantages in being relatively simple to set up, maintain, genetically manipulate and analyse. However, it is always important to critically consider whether they accurately reflect *in vivo* infection. For example, using non-epithelial and non-polarised epithelial cells e.g. HeLa, as a surrogate for epithelia should be interpreted with caution, as these cells do not always mimic the changes occurring in polarised epithelial cells and tissues during *Salmonella* infection.

Although cultured cells are often considered to be relatively homogeneous, *Salmonella* infection of cells is notoriously heterogeneous and this can limit the effectiveness of quantitative analysis unless large samples are imaged and analysed. This has highlighted the value of scaling up image acquisition and analysis e.g. by applying high content imaging techniques to mechanistic studies of *Salmonella* invasion and the role of host cell proteins in different stages of the infection cycle (83, 84). Recent studies utilising microscopy have also highlighted factors affecting heterogeneous infection of cells, including the role of 'skimming' motility in promoting invasion of cells with a prominent profile (12) and the role of vacuolar escape and subsequent replication within sub-populations of cultured epithelial cells (19). While the use of micropatterned cells may offer some benefits for standardisation of cell morphology for some infection studies (13) there remain questions regarding how closely such manipulated cells resemble *in vivo* epithelia.

When studying *Salmonella* infection of polarised epithelial cells, CLSM has distinct advantages over WFM, which is often prone to 'out-of-focus' light interfering with image resolution, even if deconvolution is applied. The redistribution of cellular proteins induced by bacterial infection has therefore been monitored by confocal microscopy in a large number of studies in both polarised epithelial cells (85-87) and in intact tissues (1, 62, 65, 87-89). It is of course not an absolute requirement to use confocal microscopy for all fluorescence imaging and excellent results can be obtained with conventional WFM, especially on flatter cultured cells (76), and spinning disk microscopes (18, 19).

Understanding how *Salmonella* trigger cellular responses relies on studying highly dynamic processes. In the early 1990's, studies using a combination of fluorescence labelling, EM and live cell imaging demonstrated that the triggering of membrane ruffling occurs soon after *Salmonella* adherence to the surface of cells (61). Several studies have demonstrated the value of live cell imaging in determining the sequence of rapidly occurring events during *Salmonella* invasion, which can be obscured by the non-synchronised nature of *Salmonella* interaction if studies are limited to the examination of cells fixed at discrete time-points during infection (72, 90). Live cell imaging is also being supported by developments in image analysis, including tracking algorithms which can semi-automate analysis of complex dynamic processes.

Imaging cellular processes in living cells can frequently reveal facets of bacterial interactions with host cells that would be impossible to determine by other means. This is especially evident when using specific fluorescent probes e.g. indicators of signalling events and GFP-tagged proteins (e.g. 8, 14, 15). An example of live cell imaging of *Salmonella* infection of GFP-actin expressing epithelial cells is illustrated in Fig 3. Such probes enable monitoring of the redistribution of proteins and other cellular changes in real time and have revealed some of the dynamic processes occurring during *Salmonella* invasion. An example from our own research is the identification of cycles of PI(3)P generation on *Salmonella*-containing vacuoles (SCVs) over several minutes, which were revealed by live cell imaging of *Salmonella* invading cells stably expressing GFP-FYVE (8). Without live cell imaging, the fact that a proportion of SCVs were

labelled with PI(3)P would have led to the assumption that this indicated a temporary location rather than the repeated cycles of acquisition and loss identified in this study (8). Similarly, live cell imaging revealed highly dynamic tubulation of SCV-associated compartments during early infection of SNX1-GFP expressing epithelial cells which were less evident in fixed cells due to their tendency to be poorly preserved during fixation (15).

Longer-term live cell imaging can also be used to study later events occurring post-invasion; including SPI-2-mediated effects on intracellular trafficking, SCV development and maturation, and bacterial division (16, 19). However, prolonging the time-course over which images are acquired can be challenging as cells are more prone to damage during such experiments, especially from photodamage if fluorescent images are acquired. Focus drift during prolonged imaging has also been a common cause of frustration but this can now be largely prevented by use of focus correction devices which detect, and adjust for, movement of a reference point such as reflection from a coverslip. As discussed earlier, WFM has distinct advantages for live cell imaging where the high intensity excitation required for CLSM is more likely to impair cellular processes. Nevertheless, advances in confocal technology e.g. spinning disk, hybrid detectors, open up further opportunities for long-term higher resolution imaging (19). The additional challenges of prolonged imaging have, however, resulted in most studies of longer term infection to date being limited to static imaging techniques (10, 11, 91).

1.2.4 *LM imaging of Salmonella properties and behaviour*

A wealth of LM techniques are available to study aspects of *Salmonella* biology aside from infection. Often these are well suited to WFM because they can be applied to bacteria within a narrow focal depth in suspension or on agar-coated coverslips. Motility, growth and septation can be examined in time-lapse studies which are often limited to phase contrast as a convenient means of locating and potentially tracking bacteria due to high contrast between the bacteria and growth medium. An example of the use of this technique to study septation of filamentous *Salmonella* (92) is illustrated in Fig 4. The combination of LM with microfluidics apparatus provides

detailed information on growth patterns under precisely controlled conditions (93). Growth of GFP-expressing *Salmonella* has also been studied by WFM to assess the impact of SPI gene expression on growth rates (94). Further, in addition to using phase contrast microscopy as a simple test of bacterial motility, flagellar dynamics have also been studied in detail following their specific labelling with fluorescent dyes (95,96).

The integrity and metabolic status of bacteria can also be assessed by fluorescence labelling. The most commonly used technique for assessing viability is the Live/Dead™ Baclight™ kit from Molecular Probes/Invitrogen. This distinguishes 'live' and 'dead' bacterial cells using two spectrally discrete fluorophores with different permeabilities. One (green) fluorophore is internalised by all cells, while the other (red) dye can only be internalised by cells with compromised membranes, the 'dead' population. Live/dead staining has been used to monitor *Salmonella* viability under adverse growth conditions (92, 97) and during infection of macrophages (98). Along with an alternative labelling method that monitors bacterial 'vitality' with an insoluble fluorescent product of electron transport chain activity (RedoxSensor™ kit from Molecular Probes/Invitrogen; 99), it has the potential to find further applications in *Salmonella* research.

2. Materials

2.1. Preparing cell monolayers

1. Most of our *Salmonella* infection studies have utilised Madin-Darby canine kidney (MDCK) epithelial cells and the details of culture methods given here are specific for these cells. Infection of other cells, both epithelial e.g. Caco-2, HeLa, Hep-2, and non-epithelial e.g. macrophages, COS cells, can be studied using similar methods.
2. Cell culture medium: Minimum Essential Eagle's Medium, supplemented with 1% (v/v) GlutaMAX (Gibco, Life Technologies Paisley, UK), 10% (v/v) fetal calf serum (Biosera, Boussens, France), 1% (v/v) non-essential amino acids (Gibco) and 100µg/ml kanamycin (Sigma-Aldrich, MO, USA) (see Note 1).

3. Glass coverslips: 13mm diameter, thickness #1 from VWR International (PA, USA), Menzel-Gläser (Braunshweig, Germany) or Corning Inc (NY, USA) (see Notes 2 and 3).
4. 75% (v/v) ethanol in dH_2O.

2.2 Infecting cell monolayers for immunofluorescence microscopy

1. Luria-Bertani (LB) broth and LB agar plates e.g. from Merck Millipore (MA, USA).
2. *Salmonella* strains grown overnight in LB broth, diluted 1:100 in fresh LB and grown in a shaking incubator at 37ºC for 3.5-4.0 hours, 150rpm (see Note 4).
3. Modified Krebs' buffer: 137 mM NaCl, 5.4 mM KCl, 1 mM $MgSO_4$, 0.3 mM KH_2PO_4, 0.3 mM NaH_2PO_4, 2.4 mM $CaCl_2$, 10 mM glucose and 10 mM Tris, adjusted to pH 7.4 at 37ºC with HCl (see Note 5).

2.3 Immunofluorescence microscopy

1. Phosphate buffered saline (PBS), pH 7.4.
2. Fixative: 2% (w/v) paraformaldehyde (PFA) in PBS (see Note 6).
3. Permeabilisation buffer: 0.3% (v/v) Triton X-100 in PBS.
4. Primary antibody: Either a specific monoclonal or polyclonal antibody generated against an antigen of interest, or an antibody fragment in some cases.
5. Secondary antibodies: Commercial fluorochrome-conjugated antibodies specific for the primary antibody (see Note 7).
6. TRITC-conjugated phalloidin from Sigma (see Note 8).
7. Mounting media: We use Vectashield® mounting media for preservation of fluorescent labelling, usually employing the version supplied with DAPI (Vector Laboratories Inc, CA, USA) (see Note 9).
8. Clear nail varnish.
9. Fluorescence microscope with appropriate fluorescence filters: Leica DMLB2 (Leica, Mannheim, Germany (see Note 10).

2.4 Confocal laser scanning microscope (CLSM)

1. CLSM: Leica TCS SP5 AOBS attached to a Leica DMI6000 inverted epifluorescence microscope (see Note 11).

2.5 Live cell imaging

1. Modified Krebs' buffer (see above and Note 5)
2. Imaging dishes: 35mm diameter (MatTek Corporation, MA, USA)
3. Microscope system and hardware: The systems we routinely use are based on Leica DMIRB inverted microscope. One has a Hamamatsu ORCA ER (12-bit CCD) camera and Prior Scientific filter wheel and shutters. The other has a Photometrics HQ2 camera, a Sutter DG5Plus illumination device, Ludl filter wheels and a PiFoc piezo focus. (See Note 12).
4. Image acquisition software: Volocity™ (Improvision/Perkin Elmer, Coventry, UK) or Metamorph (Molecular Devices, Sunnyvale, CA, USA) (see Note 12).
5. Microscope incubation chamber from Solent Scientific (Segensworth, UK) (See Note 13).

2.6 Agar-disk imaging technique

1. 13mm glass coverslips (thickness #1; VWR).
2. 75% (v/v) ethanol.
3. 90mm diameter petri dish (Greiner Bio-one, Frickenhausen, Germany).
4. LB agar.
5. *Salmonella* strains grown as required (see above and note 3).
6. Metal imaging coverslip chamber for 22mm and 24mm coverslips (custom made).
7. 22mm glass coverslips (thickness #1; VWR)
8. Microscope system and hardware: Leica DMIRBE inverted microscope (Leica Microsystems, Mannheim, Germany) with a CCD camera (Hamamatsu) (see Notes 11 and 12).

2.7 Preparation of bacteria for scanning electron microscopy

1. PBS, pH 7.4.
2. 13mm glass coverslips (thickness #1).
3. Fixative: 2.5% (v/v) glutaraldehyde in 100mM phosphate buffer pH 7.4.
4. Ethanol: 25, 50, 75, 90 and 100% (v/v) in dH_2O.
5. Hexamethyldisilazane (Sigma-Aldrich, Poole, Dorset, UK).
6. SEM specimen stubs 0.5" (Agar Scientific, Stansted, UK).
7. Specimen carbon adhesive tabs 12mm (Agar Scientific, Stansted, UK).
8. Gold sputter-coater: Emitech K575x (Quorum Technologies, Lewes, UK).
9. Scanning electron microscope: FEI Quanta 400 SEM (FEI, Eindhoven, Netherlands).

3. **Methods**

3.1 Preparing cell monolayers

1. Place glass coverslips between two pieces of tissue and spray with 75% ethanol to sterilise. When dry, use forceps sterilised in 75% ethanol to transfer the coverslips into the appropriate well plate (see Note 14).
2. Seed coverslips in well plates (or filters as appropriate) with epithelial cells. The number of cells used depends on the experimental requirements (see Note 15).
3. Incubate at 37°C in humidified atmosphere with 5% (v/v) CO_2 until required confluence reached – generally 2-4 days.

3.2 Infection of cell monolayers for immunofluorescence microscopy

The following protocol includes volumes and other details specific for infection of cells grown on 13 mm coverslips. These would need to be adjusted for cells grown on other substrates.

1. Wash cells three times with 1ml pre-warmed Krebs' buffer, with the final wash media remaining in the well. Incubation for 10-15 min at 37ºC allows equilibration of the cells in the buffer.
2. Add 50µl of the log phase *Salmonella* culture to the wells to give a multiplicity of infection (m.o.i) of approximately 50 (see Note 16).
3. Incubate cells for the required time at 37ºC (see Note 17).
4. To remove non-adherent bacteria, extract each coverslip from the well plate with forceps and wash, with moderate agitation, in a beaker of PBS.
5. Place the coverslip in a well of a 12-well plate filled with 1ml of 2% PFA, and leave to fix for 45 min (or longer) at 4ºC.

3.3 Fluorescence microscopy

There are multiple protocols that may be employed for immunofluorescence microscopy depending on which components of the cell/ bacteria are to be localised. We introduce two representative protocols which we routinely use; the first for localisation of *Salmonella* and F-actin, the second to measure *Salmonella* invasion by separately labelling the adhered and entire bacterial populations associated with cells. In each case, volumes refer to labelling of cells on 13mm coverslips. Volumes should be increased as required for larger coverslips or permeable supports.

3.3.1 Staining F-actin and *Salmonella*

1. After fixation in PFA for at least 45 min (overnight if convenient), wash the coverslips in a beaker of PBS and place in a well containing 1ml of 0.3% (v/v) Triton X-100 for 10 min to allow permeabilisation (see Note 18).
2. Wash coverslip again in PBS and place in a well of 1ml PBS.
3. Remove excess PBS from the coverslip by blotting with tissue paper (see Note 19) before transferring to an empty well.

4. Incubate cells with 50µl of primary antibody, in our case goat anti-*Salmonella* CSA-1 antibody (Kirkegaard and Perry Laboratories, MD, USA) diluted 1:200 in PBS, for 45 min at room temperature (RT). Replace the lid on the plate to prevent drying (see Note 19).
5. Add a little PBS to each coverslip to aid its removal from the well plate with forceps. Wash each coverslip in PBS and place in a well of 1ml PBS, before transferring to an empty well.
6. Incubate cells with 50µl of secondary antibody (we use Alexa Fluor® 488 anti-goat antibody from Molecular Probes, Life Technologies) and TRITC-conjugated phalloidin (Sigma).diluted in PBS, for 45 min at RT in the dark (replace lid on the plate to prevent drying, and cover with foil to limit photobleaching) (see Note 20).
7. Remove coverslip from the well plate, wash in PBS, wipe the back of the coverslip and blot with tissue to remove excess liquid.
8. Mount coverslips by placing cell side down on microscope slides with a small drop of mounting media (we generally use Vectashield® mounting media with DAPI; see Note 9). Remove excess mounting medium by gently overlaying tissue on top of the mounted coverslips.
9. Affix the edges of the coverslip with a minimal amount of clear nail varnish and allow to dryfor at least 15 min and/or store at 4°C, in order to seal and hold the coverslip in place.
10. Store slides in the dark prior to examination in order to reduce photo-bleaching.
11. Examine cells using a fluorescence microscope with the appropriate filters and objective lens (see Note 10).

3.3.2 Differential antibody staining of adhered/invaded *Salmonella* (see Note 21)

1. After fixation in PFA, wash coverslip in beaker containing PBS and place in a well containing 1ml PBS, before transferring to an empty well (Note 19).
2. Incubate cells with 50µl of primary antibody (anti-*Salmonella* CSA-1 antibody from Kirkegaard and Perry Laboratories) diluted 1:200 in PBS, for 45 min at RT. As no permeabilisation step has been performed, only the *Salmonella* adhered to the cell surface

are accessible to antibodies at this stage. Place the lid on the well plate to prevent drying (see Note 20).

3. Remove coverslip from the well plate, wash in PBS and place in a well of 1ml PBS, before transferring to an empty well.
4. Incubate cells with 50µl of secondary antibody (we use Alexa Fluor® 555 antibody from Molecular Probes, Life Technologies) diluted 1:200 in PBS, for 45 min at room temperature. During this incubation and all subsequent steps, replace lid on the plate to prevent drying and cover with foil to protect fluorophores from bleaching.
5. Remove coverslip, wash in beaker of PBS and place in a well containing 1ml of 0.3% (v/v) Triton X-100 for 10 min to permeabilise the plasma membrane.
6. Remove the coverslip from the well plate, wash in PBS and place in a well of 1ml PBS, before placing in an empty well.
7. Incubate cells again with 50µl of anti-*Salmonella* antibody (as above) diluted 1:200 in PBS, for 45 min at RT. This time both adhered and invaded *Salmonella* will be accessible to antibodies allowing enumeration of all *Salmonella* associated with cells.
8. Remove coverslip from the well plate, wash in PBS and place in a well of 1ml PBS, before transferring to an empty well.
9. Incubate cells with 50µl of secondary antibody conjugated to a different fluorophore (we use Alexa Fluor® 488 anti-goat antibody) diluted 1:200 in PBS, for 45 min at RT. Using a different fluorophore at this stage to label the total population allows the adhered bacteria to be visualised as a separate population, enabling enumeration of adhered and invaded *Salmonella*.
10. Remove the coverslip, wash in PBS, wipe the back of the coverslip and blot edge with tissue to remove excess liquid.
11. Mount coverslips cell side down on microscope slides using a drop of mounting media (we use Vectashield® mounting media with DAPI, see Note 9). Remove excess mounting medium by gently overlaying tissue on top of the mounted coverslips.

12. To seal and hold the coverslip in place, paint edges of coverslip with a minimal amount of clear nail varnish and leave to dry for 15 min and/or store at 4°C excluding light.
13. Examine cells with a fluorescence microscope using the appropriate filters and objective lens (see Note 10).

3.4 Confocal laser scanning microscopy (CLSM)

Although we have provided a protocol for imaging triple-labelled *Salmonella*-infected MDCK cells using a Leica CLSM system (see note 10) as used to generate Fig. 2, the general principles described are applicable to other CLSM systems.

1. Select 63X objective lens (glycerine immersion; 1.3 NA).
2. Examine the slide to locate suitable area for imaging. At this stage it is worth taking care to ensure the image is as clear as expected by eye (see Note 22).
3. Select appropriate zoom factor as required (see Note 23).
4. The confocal aperture (or 'pinhole') is normally left at the default setting (equivalent to 1 Airy Unit) to optimise resolution (see Note 24).
5. Select averaging e.g. averaging 3 frames as in Fig. 2, to reduce the effect of detector noise (see Note 25).
6. Select appropriate laser power for each fluorophore to allow images of these brightly stained cells to be acquired, with HyD detector settings preferably below 80% to reduce detector noise. With the CLSM system used, laser powers of <10% generally provide sufficient signal (see Note 26).
7. Using the 'TRITC' setting for red fluorophores, locate and select the top and bottom extremes of the cell. This allows a stack of images to be acquired at user-defined intervals within the selected limits (see Note 27).
8. After adjusting AOTF setting and PMT voltage for each fluorophore (Alexa 555, GFP and DAPI) and phase contrast (or brightfield or DIC) if required, save these settings to allow sequential imaging of the three fluorophores (see Note 28).

9. Choose option for sequential imaging and import previously defined Alexa 555, GFP and DAPI settings to allow switching between three excitation wavelengths during image capture (see Note 29).
10. Acquire image sequence and save as a stack of TIFF files (the default save option), which can be easily exported to other software.

3.6 Live cell imaging - time-lapse phase contrast microscopy

Although this protocol refers to the phase contrast imaging we routinely perform to examine propagation of membrane ruffles (Fig 3), the automated imaging can be modified to include fluorescence imaging e.g. GFP and/or other fluorophores or for differential interference contrast (DIC) imaging (see Note 30). The live cell imaging described here can be performed using a reasonably low cost system but faster switching and focussing, and more sensitive cameras are also available (see Note 11).

1. Make sure the microscope incubator has been running for at least 30 min, and preferably longer, to allow required temperature to be achieved and stabilised.
2. Wash imaging dishes which cells have been grown in (as described in section 3.1) twice with warm (37°C) modified Krebs' buffer.
3. Pre-warm imaging dish within microscope incubator. The time taken to move the dish between rooms will be enough for its temperature to drop so it is best to allow 5-10 minutes on the microscope stage before imaging.
4. Examine cells under phase contrast or GFP optics and select a suitable area for imaging.
5. Open Improvision Volocity software and a previously used automation for the acquisition of images using hardware described in Note 12. We typically set up the automation to capture images at three focal depths (at 1.5µm increments to ensure data is acquired at correct level and allowing for the possibility of minor stage drift) at 10 second intervals over a 20-30 minute time course. Run the automation prior to the start of imaging to allow for trouble-

shooting and to optimise the quality of the images obtained, for example by adjusting focus and lamp intensity.

6. Adjust automation to include specific exposure times and focus increments for all channels. These could be standard protocols for established techniques or ones set according to the specific requirements of the sample (see Note 31).
7. When ready to start imaging, begin the automation and then, after 1-2 min, add 50µl of log phase culture to the Krebs' buffer in the coverslip holder (see Note 32).
8. Images can be exported for processing and analysis in the Volocity format to an off-line workstation running Volocity core software. Alternatively images can be exported as a stack of TIFF images for processing with other software.

3.7 Imaging live bacteria - agar-disk imaging technique

This method can be used to examine bacterial suspensions, for example to assess GFP expression, viability or to monitor division (as in Fig. 4).

1. 13mm coverslips are sterilised by placing them between two pieces of tissue and spraying with 75% (v/v) ethanol. When dry, forceps sterilised in 75% ethanol should be used to transfer the coverslips to a sterile petri dish.
2. To make agar disks, 200µl of molten LB agar – supplemented with antibiotics if appropriate – is applied to the centre of each 13mm coverslip and allowed to set (Fig. 5).
3. To prepare a sample for imaging, spread 7µl of fixed or live bacterial culture onto a 13mm agar disk and allow it to dry for 1 min at room temperature.
4. Prepare a metal imaging chamber to receive the agar disk by mounting a 22mm glass coverslip over the rubber O-ring and screwing the base of the imaging chamber tightly against the glass to form a seal.
5. Using sterilised forceps to grasp the glass base of the agar disk, invert it into the imaging chamber with the bacterial sample resting against the large glass coverslip, (Fig. 5).
6. To capture phase contrast images of the bacterial sample, mount the imaging chamber onto a Leica DMIRBE inverted microscope with an appropriate 63X or 100X oil-immersion

lens and a CCD camera (as above and see Notes 10 and 33). All images and time-lapse sequences should be captured at maximum resolution if possible.

3.8. Preparation of infected cells for scanning electron microscopy.

This protocol describes how to prepare *Salmonella* cultures for visualisation by SEM, however the general principles described here are also applicable to other SEM systems. This protocol is a quick method that is adequate for our requirements but improved results may be obtained in some cases using protocols involving additional fixation steps and/or critical point drying.

1. Following infection of monolayers as described in section 3.2 (Note 17), place coverslips in individual wells filled with 2% glutaraldehyde in 100mM phosphate buffer pH 7.4 and leave to fix overnight at 4^{o}C.
2. To dehydrate the sample, incubate fixed coverslips sequentially in 25, 50, 75, 90, 100 and 100% ethanol for 20 min at each concentration at room temperature.
3. In a fumehood overlay coverslips with 1ml hexamethyldisilazane and incubate for 1h at room temperature.
4. Remove coverslips and transfer to a new 12-well plate and allow residual solvent to evaporate at room temperature.
5. Following solvent evaporation, attach coverslips to SEM stubs with adhesive carbon tabs and coat with gold/palladium using an Emitech sputter coater.
6. Store samples in airtight conditions until examination with an FEI Quanta 400 SEM.

4. Notes

1. Media recipes differ for other cells. Cell culture media and supplements are available from many suppliers.
2. Coverslips are available in a range of thicknesses. Most often we use thickness #1 (130-170 μm) and do not go to any additional lengths to determine the precise thickness of individual coverslips, despite known variability in thicknesses. Microscope manufacturers often specify

170 nm as the optimum coverslip thickness for performance of their objective lenses; so some researchers prefer to purchase thickness #1.5 (160-190 μm) as a result. It should, however, be remembered that many of these will be too thick to allow optimal resolution. Small differences in coverslip thickness are unlikely to significantly affect image quality in most applications, but where optimal resolution is needed e.g. to ensure optimal precision in deconvolution, some researchers will check the thickness of coverslips. High performance objective lenses often have correction collars that should be adjusted to the correct coverslip thickness and it is advisable to make fine adjustment to these collars while viewing the effects on image clarity to ensure correct positioning. Multi-well slides e.g. Lab-Tek, also provide a convenient means of separately treating multiple cell samples on a single slide. At the end of the experiment the chambers are removed and a coverslip placed onto the slide. Cells can also be grown on conventional plasticware if low magnification is sufficient or in multiwall plates with coverslip-quality glass bottoms if high resolution is required.

3. MDCK cells, like some other epithelial cell lines, can form functionally polarised monolayers when grown on permeable supports. The most commonly used permeable culture inserts include Transwell™ (Corning) and Anopore™ (Nalge Nunc International, NY, USA). Epithelial cells able to form polarised layers should be grown on such permeable supports when the effects of *Salmonella* on epithelial properties, such as transport and barrier functions, are to be investigated.
4. Although we mostly use this method to obtain mid-log bacteria with maximal invasiveness, other growth parameters can also be used as required.
5. We use Krebs' buffer as a convenient physiological buffer that avoids the requirement for CO_2 that comes with using bicarbonate-buffered media. Alternatives, which are especially useful for longer-term infection studies, include conventional cell culture media or bicarbonate-free versions of these that incorporate alternative buffering to avoid the requirement for CO_2. For fluorescence microscopy, avoidance of Phenol Red (which is included in standard cell culture media as a pH indicator) is advisable as this causes substantial background fluorescence.

6. PFA causes less autofluorescence than glutaraldehyde. PFA should be prepared fresh or can be stored in frozen aliquots for many applications. We don't use commercial formaldehyde solutions due to impurities. PFA fixation works well for a large proportion of antigens but in some cases solvent fixation e.g. methanol, acetone, or mixtures of these, gives superior results. Solvent fixation also has the advantage that it permeabilises cells and thus eliminates the requirement for this additional step.
7. Indirect immunofluorescence is more commonly used than direct fluorophore conjugation of primary antibodies for a number of reasons including usual scarcity of primary antibodies and the signal amplification and flexibility in fluorophore selection provided by the indirect approach. Secondary antibodies are generally raised in larger animals and are available in a wide variety of fluorophore-conjugates (excited from UV through to red) from suppliers including Jackson ImmunoResearch Laboratories, Molecular Probes/Invitrogen, and Sigma. In recent years the first generation fluorophores such as fluorescein and rhodamine derivatives have largely been superseded by improved fluorophores, including the extensive range of Alexa Fluor® dyes (350, 488, 555, 594, 633 etc) and the cyanine dyes (Cy2, Cy3 and Cy5) which have higher quantum yield (brightness) and improved photo-stability.
8. We generally use TRITC-phalloidin, but alternative fluorophore-conjugated versions e.g. Alexa Fluors®, FITC, Cy5, are available from Sigma, Molecular Probes/Invitrogen etc.
9. We routinely use Vectashield® supplied with DAPI, unless we specifically want to avoid an additional UV-excited dye interfering with labelling in that part of the spectrum or when we use the far-red alternative TOPRO-3 to avoid the cross-talk between DNA labelling and other fluorophores which is unavoidable when using DAPI. The main advantage of using DAPI-containing mountant is to avoid an additional labelling step and the need to prepare toxic solutions of DAPI or alternative DNA labels. Many researchers instead mount with Mowiol™ containing n-propyl gallate as an anti-fade, which sets hard unlike the Vectashield® we use. A hard-set version of Vectashield® is also available from Vector Laboratories. Other alternative mountants available from other sources include SlowFade Gold™ and ProLong™ from Molecular Probes/Invitrogen. Anti-fade mountants can vary in their effectiveness against

photobleaching, and also in the initial intensity of fluorescence. We have not performed a thorough side-by-side comparison of their properties. It is also important to bear in mind that mounting media may not be completely compatible with all fluorophores. For example some users have reported decreased signal from Cy2 and other cyanine dyes over time when mounted in media containing phenylenediamine as an anti-fade.

10. This can be a relatively simple upright or inverted microscope with fluorescence capabilities. Usually this will have separate fluorescence filter blocks for each fluorophore and be equipped with a range of objective lenses. The major suppliers include Leica, Olympus, Zeiss and Nikon. All of these produce high quality optical instruments and choice comes down to personal preferences and practicalities. Details of the Leica upright microscope we use for examining staining are available at http://www.bris.ac.uk/biochemistry/mrccif/techspecleicadm.html.
11. We use one of a range of Leica CLSMs. The one used to acquire images in Fig 2 is a Leica TCS SP5 AOBS tandem scanning CLSM attached to a Leica DMI6000 inverted epifluorescence microscope. Further details of this and our other confocal microscopes are at http://www.bris.ac.uk/biochemistry/mrccif/equipment.html. Other suppliers of CLSMs include Zeiss, Olympus and Nikon.
12. Further details of the WFM system used to acquire images in Fig 3 are available at: http://www.bris.ac.uk/biochemistry/mrccif/equipment.html. Alternative imaging systems, including integrated acquisition and deconvolution systems are available from other companies e.g. Applied Precision, Leica, Zeiss, Olympus, Molecular Devices and Media Cybernetics. Each of these has image processing and analysis capabilities. Some researchers prefer to build their own systems, and the open source software, Micro-manager, is now a popular choice for this due to its ability to drive an expanding range of microscope hardware. Similarly, ImageJ, with its extensive range of plug-ins for image processing and analysis is a popular alternative to commercial image analysis software.
13. There are distinct advantages in sample stability (reducing focus drift) by enclosing a large proportion of the microscope in a temperature-controlled environment and ensuring temperature equilibration of cells before imaging, the advantages being more apparent with

long-term imaging. Focus control systems e.g. CRISP from Applied Scientific Instrumentation, Eugene OR, USA, can also be used to correct for any residual focus drift, which is not always possible to eliminate with temperature control devices. Cells in dishes can also be maintained at 37°C and perfused using on-stage heating devices as supplied by various companies e.g. Harvard Apparatus, Scientifica, Life Imaging Services, Bioptechs. If using bicarbonate-buffered media it is necessary to have a means of enriching the environment around the cells with CO_2.

14. For fluorescent labelling of fixed cells we favour 13mm coverslips in 12-well tissue culture plates. For live cell imaging applications we grow cells on 35mm imaging dishes with 14mm glass coverslips.
15. We typically seed 13mm coverslips with 1ml of $1x10^5$ cells/ml or 1ml of $0.66x10^5$ cells/ml so that after two and three days, respectively, we obtain cells with ~80% confluence. The same dilutions but 2ml volumes are used for 35mm MatTek dishes.
16. This gives a high infection rate which is ideal for live cell imaging and some other experiments. Decreased m.o.i. can be used to provide more realistic infection levels and are preferable for longer-term infection studies. When comparing strains it is important to check that growth characteristics are similar to ensure infection is comparable i.e. similar numbers of bacteria at equivalent growth state. This can be measured by determining absorbance at 600nm with volumes adjusted to match m.o.i. for different cultures or more accurately by determining colony forming units (c.f.u.).
17. We routinely examine time points between 5 and 60 min. When later time points after infection are to be examined we use a ‘pulse-chase’ protocol involving initial 15 min infection followed by removal of external *Salmonella*.
18. Alternative detergents e.g. saponin are used in some protocols. Permeabilisation is not required if solvent-fixation is employed.
19. To remove excess PBS from coverslips prior to labelling, dry the back of the coverslip with fine tissue paper and remove excess liquid remaining on the sample surface by tilting the coverslip and holding the edge of the coverslip with tissue paper. This step is used between all staining steps but has been omitted from elsewhere in the protocols for brevity.

20. It is important to stress that the labelling methods described here and in section 3.3.2 are minimalist protocols used for speed and convenience. We know the labelling will be bright and we are not overly concerned with the presence of some non-specific labelling. This basic protocol does not include any steps to quench autofluorescence or to block non-specific binding. Several alternative modifications could be employed to decrease non-specific labelling, the most common being to include BSA or serum from unrelated species (preferably including the same species as that in which secondary antibodies were raised) before and/or during antibody incubations. For example, we have frequently used pre-blocking with 1% (w/v) BSA (or 5-25% serum) in PBS as well as addition of 0.1% BSA (or 5-10% serum) in the antibody diluents.
21. This is just one alternative method for differentially labelling adhered and invaded *Salmonella*, an alternative is the use of methanol fixation as discussed in section 1.2.2. Although the resulting staining might be cleaner if blocking steps were introduced, this simplified method has proved adequate for our requirements.
22. It is important to optimise image clarity during visual inspection. It is useful to check that there is no additional factor, such as contamination of oil or incorrect objective setting that will compromise image quality. With thicker specimens e.g. tissues that are more likely to be examined by CLSM than WFM, it can be much harder to determine if the image is optimal because of the level of out-of-focus fluorescence, so it may be worth checking this with a pre-prepared, familiar and clear specimen such as a standardised slide.
23. In this case, in order to meet the requirements for optimal resolution imaging (59) a zoom factor was selected to obtain 43nm pixels at default size 1024x1024 pixel density. It is not always necessary to optimize XY resolution to this extent and it is worth bearing in mind that increasing zoom also increases the light dose per pixel so may be accompanied by increased photo-bleaching, and, in the case of live cell imaging, phototoxicity.
24. The confocal pinhole can be opened if required to increase sensitivity, which is especially useful for live cell imaging. Significant increases in signal can be achieved with only minor loss of axial resolution. It is also worth considering the light-collecting properties of the objective

lens when applications require optimal signal e.g. live cell imaging. For example, signal intensity achieved with the oil-immersion lenses available on our CLSM system decreases with increasing magnification such that users will often prefer to use a 40X or 63X objective lens and zoom rather than choosing 100X objective.

25. Depending on the characteristics of the detector, and the amplification applied, there may be additional benefits in noise reduction to be gained from averaging more than 3 frames. It should be taken into consideration that more averaging comes at the expense of increasing the time taken to acquire stacks, in addition to increasing the light dose received by the sample, with resulting risk of photo-damage. For live cell applications averaging is often limited to fewer frames or completely avoided due to considerations of phototoxicity, as well as movement-based artefacts. Most CLSM systems will now allow line averaging rather than frame averaging, which is particularly useful where movement is likely to affect information within acquired images, as the time between repeated line scans is negligible compared to the frame interval. We still prefer frame averaging for fixed samples as line averaging can result in decreased signal intensity.
26. Today's CLSM systems generally have acousto-optic tuneable filters (AOTFs) to allow rapid and independent adjustment of illumination intensity from individual laser lines. In the case of fixed (and anti-fade mounted) specimens, the laser power is less critical than for live cell imaging, where it is often necessary to use higher PMT voltages to enable laser power, and hence photo-bleaching/damage, to be minimised. We use a colour LUT that clearly identifies pixels with intensities 0 or 255 (extremes of 8-bit range) by displaying them as green or blue, respectively. In the case of CLSM there is less advantage to be gained by using 12-bit imaging because, in contrast to WFM, the number of photons detected for each pixel is usually rather small. The image in Fig 2 was acquired using the Ar laser (488nm) at 10% and 561nm laser at 2% with detection set at 493-549nm for GFP and 569-651nm for Alexa 555. GaAsP (HyD) detectors were used for both channels, at 65% and 74% gain.
27. Of course it is not always necessary to obtain image stacks, sometimes a single image or at most a small number of sections are all that are required if a limited part of the cell depth

contains the required information. In the case of the image shown in Fig 2, the full depth amounted to an approximately 6 μm stack and step size was set at 126 nm to optimise data acquisition (Nyquist sampling). More often, larger step sizes are selected e.g. 500nm, to limit the time required to obtain images, and thereby photobleaching, as well as to limit imaging time, light dose and file size.

28. This approach has distinct advantages over acquiring images simultaneously, as it usually avoids the cross-talk between fluorophore signals that is almost inevitable if all three fluorophores are excited simultaneously. For example, DAPI emission spectrum overlaps with those of both Alexa Fluor®488 and 555, while the Alexa 488 emission is also likely to contaminate the Alexa 555 channel when labelling with the green fluorophore is relatively intense. With careful selection of fluorophores and balanced labelling intensity, two or more fluorophores can be imaged simultaneously without significant cross-talk, with resulting savings in imaging time.
29. In this case the image stacks were acquired using 'line-by-line' sequential imaging where the AOTF is controlled to rapidly switch between excitation lines for every line scanned such that the fluorophores are imaged at millisecond intervals and appear simultaneously on the display panel. Other parameters such as PMT settings, the wavelength range detected, and pinhole size can be adjusted between fluorophore settings only in the other, slower, and sequential modes i.e. 'between frames' and 'between stacks' in the Leica confocal software.
30. DIC imaging affords enhanced contrast for some samples compared to phase contrast, although in our hands we have preferred images acquired by phase contrast to monitor ruffle propagation and internalised *Salmonella*. Automated imaging of fluorescence e.g. GFP, alongside phase contrast is somewhat more straightforward than with DIC, as the DIC optical components reduce detected fluorescence signal intensity. This can be avoided by using a motorised filter cube to switch between DIC and fluorescence filter blocks or by placing the DIC analyzer within emission filter wheel, though there will be an additional time delay introduced into the time course using this approach. The loss of sensitivity using DIC simultaneously with fluorescence is much greater than the small loss of light transmission caused by the phase ring

within phase contrast objectives. Phase contrast also has advantages in facilitating clear discrimination of internalised bacteria which are often more difficult to detect in DIC images.

31. Live cell imaging automations can be customised to switch between, for example, GFP and phase contrast imaging. This can be used to examine the dynamics of intracellular GFP-tagged probes (as in Fig 3) or GFP-expressing bacteria alongside morphological responses to infection. Automated shuttering of the light source is beneficial to decrease likelihood of photobleaching or phototoxicity compromising data acquisition.
32. In order to compare one strain or growth condition with another it is necessary for the cultures to have been incubated for the same length of time. Therefore, we set up cultures at 45 min intervals, and subsequently the imaging of each culture is timed to allow 20-30 minute time courses to be acquired while also allowing time to set the system up for capturing the next time course.
33. This methodology can also be adapted to allow visualisation of GFP-expressing bacteria or those stained using a fluorescence stain, for example DAPI or the Molecular probes LIVE/DEAD® *bac*Light™ viability kit for microscopy (Invitrogen Life Technologies, Paisley, UK). To view fluorescent bacteria using this method, stain the bacteria according to the manufacturer's instructions and apply 7µl of stained sample to the agar disk. To prevent bleaching of the fluorescent sample, allow the sample to dry onto the surface of the agar in the dark. In order to visualise the bacterial sample, use a fluorescence microscope with appropriate filters (see note 10).

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Figure Legends

Fig 1. Scanning electron microscopy (SEM) used to investigate surface structure of *Salmonella* biofilms (panel A) and membrane ruffles induced on epithelial cells during infection (panel B). Dense *Salmonella* community architecture includes prevalent filamentous material after 48h growth on plastic coverslips (A). Infection of MDCK cells for 15 min with *Salmonella* leads to formation of prominent membrane ruffles (B). Panel A: 3.5x2.8 µm. Panel B: 9x7.2 µm.

Fig 2. Comparison of WFM and CLSM imaging and the effects of deconvolution on image clarity. Images of *S.* Typhimurium-infected GFP-actin expressing MDCK cells stained with anti-*Salmonella* antibody and Alexa 555-conjugated secondary antibody to localise bacteria relative to actin cytoskeleton with both channels presented in a single monochrome image. Panel A: Representative image from a stack of WFM images acquired at 250nm intervals with a 100X oil immersion lens (NA 1.4) throughout the cell depth and beyond with parameters recommended by Huygens software (59). Panel B: After deconvolution using Huygens software (and a calculated PSF), a prominent increase in clarity is observed due to removal of out-of-focus fluorescence and increased spatial accuracy within this optical section. Despite the very clear improvement in image clarity, which is especially evident for *Salmonella* localisation (arrows), deconvolution has been less effective in removing out-of-focus information from some parts of the actin cytoskeleton due to its highly complex distribution. Panel C: A representative CLSM image from a stack of 160nm steps acquired with Leica SP5 AOBS system and a 63X glycerine-immersion lens, with pixel size (43nm) optimised for Nyquist sampling (59) by using the zoom function. Note the improved image clarity compared with the WFM in panel A that is especially prominent for the more diffuse actin distribution (arrows). This image nevertheless suffers from a common problem with CLSM imaging, that of low signal-to-noise and a tendency for high resolution images to appear ‘speckly’. Panel D: CLSM image as shown in panel C following deconvolution (Volocity software) improves resolution

and reduces noise. Field of view approx 42x32μm (panels A and B) and approx. 28μm x 21μm (panels C and D).

Fig 3

Use of time-lapse phase contrast and fluorescence WFM to examine membrane ruffle propagation and development in GFP-actin expressing MDCK cells. Representative phase contrast (top row) and fluorescence (bottom) images of a membrane ruffle generated by wild type *S*. Typhimurium (SL1344) is shown at distinct points in ruffle development during a 20 minute time course. Timestamps on each image indicate relative time compared to the first image in which the bacterium (arrows) responsible for inducing this membrane ruffle attached to cells (0s). Increased fluorescence due to GFP-actin concentration as *Salmonella* induce membrane ruffling is evident by 80s following attachment (arrow) and very prominent at 100s and later time points (arrows), when ruffling is also clearly evident in phase contrast images (arrows). Scale bar = 5 micron.

Fig 4

Septation of filamentous *Salmonella* monitored by phase contrast microscopy following relief from filament-inducing conditions. *Salmonella* on agar disks imaged every 2.5 minutes to follow the septation process. Figures refer to time intervals (minutes) for each figure. Scale bar represents 10μm.

Fig 5

Agar disk imaging technique. A. 13mm agar coverslips are prepared by spreading 200μl molten LB agar onto sterile 13mm glass coverslips. Once set, 7μl of bacterial sample is spread onto the surface of the agar and allowed to dry for 1min. B. Using forceps, a 22mm glass coverslip is mounted onto a metal imaging chamber over the O-ring seal. C. The base of the metal imaging chamber is screwed on tightly against the glass coverslip to form a seal. D. Using forceps, the agar disk is inverted into a metal imaging chamber, with the bacterial sample resting against the glass coverslip.

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