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Microreview

Progression of bacterial infections studied in real time – novel perspectives provided by multiphoton microscopy

Lisa E. Månsson,[1] Keira Melican,[1]
Bruce A. Molitoris[2,3] and Agneta Richter-Dahlfors[1]*
[1]*Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, S-171 77 Stockholm, Sweden.*
[2]*Division of Nephrology, Department of Medicine and*
[3]*Indiana Center for Biological Microscopy, Indianapolis, IN 46202, USA.*

Summary

The holy grail of infection biology is to study a pathogen within its natural infectious environment, the living host. Advances in *in vivo* imaging techniques have begun to introduce the possibility to visualize, in real time, infection progression within a living model. In this review we detail the current advancements and knowledge in multiphoton microscopy and how it can be related to the field of microbial infections. This technology is a new and very valuable tool for *in vivo* imaging, and using this technique it is possible to begin to study various microbes within their natural infectious environment – the living host.

Introduction

One of the main challenges of current research is to understand what happens when host cells first encounter bacterial pathogens. What is the kinetics of the immune response and how are signals transduced from the host cell which leads to the induction of a synchronized tissue response? Previous research in bacterial pathogenesis has largely been limited to model systems consisting of cultured cells, isolated primary cells or freshly isolated tissue segments. Using these simplified *in vitro* or *ex vivo* systems, experiments can be designed to address well-defined and precise hypothesis in a reproducible manner. The lack of supporting tissues, including the vasculature, nervous system and extracellular matrix, generates, however, a concern that these experimental systems may not accurately represent the physiological situation (Dunn *et al*., 2002). In contrast, *in vivo* animal models convey the great advantage of including all the interacting players, providing a model relevant for studies of human disease. The complexity of these models can, however, result in somewhat ambiguous answers, answers that may include a number of factors not previously taken into account (Wiles *et al*., 2006). Thus, to grasp the full picture of the processes underlying bacteria–host interactions, there is a need for *in vivo* based methods to support and expand upon the current *in vitro* approaches.

Multiphoton microscopy is a non-invasive technique developed by biophysicists and adapted by biologists to study dynamic processes within living organs. The first application of multiphoton microscopy to study live cells was presented by Denk *et al*. (1990), and was followed a few years later by a demonstration of the potential of this technique for *in vivo* studies of brain (Denk *et al*., 1990; 1994). Multiphoton microscopy has since been utilized to explore a broad array of neurobiological phenomena, including the dynamics of Ca^{2+} fluctuations in individual synapses and the role of astrocytes in normal and pathological brain (Svoboda and Yasuda, 2006; Tian *et al*., 2006). The potential of multiphoton microscopy for high-resolution and high-sensitivity fluorescence microscopy of intact tissues has, over recent years, been adopted by researchers in a variety of fields including studies of tumour vascularization (Brown *et al*., 2001), kidney physiology (Molitoris and Sandoval, 2005), embryonic development (Squirrell *et al*., 1999), T-cell behaviour (Bousso and Robey, 2004), heart function (Rubart, 2004), the immune system (Cahalan and Gutman, 2006) and skin diagnostics (Schenke-Layland *et al*., 2006). The tremendous potential of multiphoton microscopy for studies within the field of infection biology has only recently begun to be realized (Chieppa *et al*., 2006; Mansson *et al*., 2007). In this

Received 2 May, 2007; revised 21 June, 2007; accepted 25 June, 2007. *For correspondence. E-mail Agneta.Richter.Dahlfors@ki.se; Tel. (+46) 468 52487425; Fax (+46) 468 342851.

Table 1. Relative comparison of fluorescence imaging techniques.

	Scanning confocal	Spinning disc	Two-photon
Applicable to live animal	(+)	++	+++
Applicable to live cells	+	+++	++
Applicable to fixed tissues	+++	+++	+++
Tissue depth	++	+	+++
Tissue damage/bleaching	+++	++	+
Spatial resolution	+	+	+
Speed	+	+++	+
Signal to noise ratio	+	++	++

See text for details of advantages and disadvantages of different methods.

review we describe how multiphoton microscopy can be used to study tissue responses to a variety of bacterial pathogens and their virulence factors, where the spatiotemporal resolution is the key to understand the inflammatory processes.

Fluorescent imaging techniques for bacteria–host interactions

Detailed studies on the organ and cell level of infected animals have, in the past, required extraction of the tissue and histopathological preparations in order to perform microscopical analysis. Traditional optical microscopy techniques, which use linear (one-photon) excitation light, cause out-of-focus blur and light scattering that greatly limits the depth of high-resolution image collection (Helmchen and Denk, 2005). Confocal microscopy improved the quality of microscopic images with the introduction of a detection pinhole that rejects all light that does not originate from the focus site. This does, however, also limit the signal strength, and may cause an inadequate signal to noise ratio (SNR). While in fixed cells SNR can be improved by increasing the power of illumination, amplified illumination may be phototoxic and increase fluorophore photobleaching during live cell imaging. Spinning disc confocal systems, where fluorescence emission is collected through a set of fixed-sized pinholes on a perforated disc (Nipkow disc), may achieve the same SNR as a single-point scanning confocal system, but only causes 1/15 of the photobleaching (Wang *et al*., 2005). The fast collection of images enabled by the spinning disc system has meant a revolution in high-speed capturing of live cells, going from seconds to milliseconds (Nakano, 2002). The system has been successfully applied to live animal infection models such as mouse ears for investigating plasmodium infection routes (Amino *et al*., 2006) and in zebra fish models to investigate the microbial flora of the gut (Rawls *et al*., 2007). The spinning disc is not, however, optimal for thicker samples with significant amounts of out-of-focus fluorescence, where the cross-talk between adjacent pinholes on the disc becomes significant. Another limitation is that the fixed size of the pinholes does not allow for optimizing resolution at different magnifications. Table 1 presents a comparative summary of the various fluorescence imaging techniques discussed in this article. Values given in the table are relative as many of the factors listed are interconnected and the values of each parameter depends on the choice of tissue and experimental set-up.

Recent developments have generated non-linear optical microscopy techniques, such as two-photon or multiphoton imaging, which are less sensitive to optical light scattering and are therefore well suited to high-resolution imaging deep into biological tissues. Two-photon fluorescence depends on two photons arriving simultaneously at the point of focus, where their energies combine to promote fluorescence excitation (Helmchen and Denk, 2005). The lack of out-of-focus excitation makes pinhole filtering of the emitted light unnecessary and increases sensitivity as all emitted light is captured. Another advantage is that the combination of the colliding photon energies renders higher energies than they would on their own. Light with lower energy, in the near infrared spectra, penetrates deeper into light-scattering tissue and is generally less phototoxic (Denk *et al*., 1990). Thus, multiphoton imaging is less harmful to tissue, an essential aspect for long-term repeated live imaging. Living tissues can be examined in detail at a depth of up to 1 mm without compromising the tissue (Theer *et al*., 2003); however, the actual depth achieved is a function of the light-scattering properties of the tissue being studied. In the kidney, for instance, depths of up to 150–200 μM are achievable.

Previously, studies aiming to analyse a sequential course of events required tedious sacrificial time point experiments, where the lack of continuity of infection within a single animal required the sacrifice of many animals at each time point. Multiphoton imaging allows the progression of events to be followed in the same animal over long periods of time, which thereby dramatically reduces animal to animal variability and the number

of animals needed in the study. The continuity of the multiphoton-based studies also circumvents the biased selection of time points when tissue is fixed for visualization by confocal microscopy, a procedure that may bias important tissue events.

In the set-up of a live multiphoton model the accessibility of the organ to the microscope is crucial. Obstructions may be strictly physical, e.g. penetrating the highly light-scattering skull to reach the brain. Neuroscientists have solved this by preparing a cranial window, surgical removal of a piece of the skull bone, or by substantial thinning of the skull, without damaging the underlying dura and cortical tissue (Denk *et al*., 1994). Artefacts and noise are also produced by subject movement due to heart beat or respiration of the anaesthetized animal. Therefore, to obtain high-resolution images and enable three-dimensional rendering of the captured site, movement has to be eliminated. Organs that can be externalized without compromising function, such as the kidney and gut, are good targets for imaging. Chieppa *et al*. (2006) recently demonstrated multiphoton imaging of infection within a small intestine which had been exteriorized with close attention paid to blood and lymph connection. The internal organization of organs in the body may also affect organ availability to the microscope or the choice of upright or inverted microscope. The presence of the liver on the left hand side of the body has pushed the left kidney further away from the bladder making the left ureter slightly longer. The longer ureter allows for easier externalization of the kidney without affecting the blood flow or function (Molitoris and Sandoval, 2005; Mansson *et al*., 2007).

Despite the numerous advantages of multiphoton imaging, there are of course many factors that must be taken into account when selecting an imaging technique. While multiphoton is far less damaging to tissue than conventional confocal scanning microscopy, when working on living tissue the effects of laser exposure must still be taken into account. Continuous imaging of a single site has, in our hands, shown some level of tissue damage. Subtle effects on cellular processes such as endocytosis appear to be the most common signs of phototoxicity and therefore the benefits of continuous imaging must be weighed against possible tissue damage. Other potential disadvantages with live animal imaging include the numerous complications involving animal movement and maintenance of physiological conditions. Maintenance of factors such as blood pressure and body temperature are crucial to the validity of the results. A drop in body temperature to just 34°C results in a drop of 50% on cortical renal blood flow, the site of multiphoton imaging (Deetjen *et al*., 1964). The choice of anaesthesia may also affect blood pressure and other physiological factors over an extended period of imaging (Conger and Burke, 1976). The benefits of live multiphoton imaging are tremendous but to maintain the most relevant physiological conditions requires a delicate balance between numerous factors, and at present results in a rather labour/time-consuming exercise requiring a variety of specialized techniques.

Fluorophores for *in vivo* imaging

To allow visualization of the progression of infection *in vivo*, the bacteria, as well as other inflammatory markers, need to be discernible through the microscope. Tissue autofluorescence can be regarded as a problem during microscopy as it decreases the SNR, but during live imaging it can be helpful to facilitate orientation in the tissue. Dyes targeting specific tissue structures are, however, crucial and the introduction of the green fluorescence protein (GFP) in the early 1990s resulted in a new era in fluorescent labelling (Chalfie *et al*., 1994). It is now possible to label and monitor the behaviour of individual proteins non-invasively and continuously. The original GFP protein, recovered from the jellyfish *Aequoria Victoria*, has been modified and several homologues have been identified in related species. Currently, a range of fluorescent proteins, spanning the entire visible spectrum (~450–650 nm), are available (Chudakov *et al*., 2005). This allows for simultaneous observation of more than one reporter in the same experiment. In addition, induced mutations have resulted in higher fluorescence intensities and an increased resistance to photobleaching. Other changes have been directed towards optimization of protein synthesis in the host cell type. The development of various refined fluorophores allows for novel applications of *in vivo* fluorescent labelling to study protein expression, interaction, activity, movement and turnover (Chudakov *et al*., 2005). Live functional and motility studies of organelles as well as whole cells, with both prokaryotic and eukaryotic origin, is also possible (Southward and Surette, 2002). Other fluorescent probes have been developed which demonstrate a spectral response upon binding ions such as calcium, allowing investigation of the fluctuations in intracellular Ca^{2+} concentrations. When working *in vivo*, however, there are certain restrictions placed upon the range of antibodies and dyes that can be used. All fluorophores must interfere as little as possible with the natural processes of the tissue. Common antibodies used to identify such things as infiltrating immune cells cannot be used *in vivo* due to the blocking or activating effect they may exert upon the cells. By combining the recent developments in fluorescence labelling techniques and *in vivo* multiphoton microscopy, real-time studies of targeted processes can be performed on cellular as well as subcellular levels in intact tissues or living animals.

Visualizing bacteria *in vivo*

To enable multiphoton microscopy of bacterial infections *in vivo*, relevant bacterial strains have to be modified to allow intravital detection. Labelling of live structures is generally obtained through systemic delivery of specific dyes or antibodies, or via endogenously produced signals, either from a plasmid-borne system or through chromosomal fusion. Antibodies directed towards the bacterial outer membrane component LPS are widely used for immunohistochemical applications, but *in vivo* distribution of such antibodies is not possible. Currently there are no prokaryotic dyes for intravital use available on the market. As bacterial expression of fluorescent proteins does not require any substrate for endogenous production, it is the method of choice for prokaryotic labelling. Typically fluorescent constructs are cloned into multicopy plasmids that are transformed into the bacteria to be used in infection studies (Hansen *et al*., 2001; Qazi *et al*., 2001; Torstensson *et al*., 2005). The variation of plasmid copy numbers in individual bacteria, however, may result in an unreliable signal. Other problems include the need for antibiotics within the animal to ensure that bacteria maintain the plasmids, which may have unwanted physiological side-effects. Finally, multicopy plasmids produce high concentrations of the fluorescent protein, which can be potentially toxic for the bacteria. Variants of the fluorescent protein GFP, such as *gfp*$^+$, have been modified to enhance brightness and reduce toxicity and subsequently are very suitable for *in vivo* imaging (Scholz *et al*., 2000). The ever-growing library of fluorescent proteins is giving a great variety of choice to researchers looking for ways to visualize bacteria. Chromosomal single-copy transcriptional fusions, where bacterial gene expression can be detected by GFP production, have been reported in both Gram-positive, e.g. *Listeria monocytogenes* (Gahan *et al*. 2001), and Gram-negative bacteria, e.g. *Salmonella enterica* (Hautefort *et al*., 2003). Hautefort *et al*. (2003) found transcriptional fusion with *gfp*$^+$ suitable for assessing prokaryotic gene expression during infection of mammalian cells. Plasmids were designed which encoded the *gfp*$^+$ gene downstream of an antibiotic resistance gene, which could be replaced by a promoter of choice. This plasmid provided a template for production of a linear PCR product which could be transfected into the bacteria and recombined onto the recipient chromosome by allelic replacement (Datsenko and Wanner, 2000).

To follow bacterial dissemination during mammalian infection by endogenous production of a fluorescent protein, a constitutively active promoter has to be identified. The P_{rpsM} promoter has been reported to have similar levels of transcriptional activity in various environments, including bacteria cultivated *in vitro* as well as replicating inside mammalian cells (Valdivia and Falkow, 1997, Hautefort *et al*., 2003). As this promoter drives the expression of ribosomal proteins, it is possible that the transcriptional activity may vary during the course of an infection as the bacteria constantly adapt their protein production to the changing microenvironment (Post *et al*., 1980). When investigating the activity of P_{rpsM} during kidney infection using a retrograde rat urinary tract infection (UTI) model, we found that only a fraction of the bacteria expressed GFP$^+$ when regulated by this promoter (our unpublished observation). This confirmed that the P_{rpsM} promoter was not suitable for *in vivo* visualization in complex organs. Stable bacterial GFP$^+$ expression was, however, obtained during UTI using the tetracycline promoter, $P_{LtetO\text{-}1}$, which was cloned in the absence of the *tet* repressor, altering $P_{LtetO\text{-}1}$ from an inducible to a constitutively active promoter (Mansson *et al*., 2007). The *LacZ* promoter has also been used to drive expression of DsRed in *Salmonella* (Avogadri *et al*., 2005). This fusion has been used to successfully visualize *Salmonella* interacting with dendritic cell balloon bodies using multiphoton microcopy (Chieppa *et al*., 2006).

The *gfp*$^+$ fusion system has shown great potential in detecting and measuring the expression of bacterial virulence factors in real time during the course of infection (Mansson *et al*., 2007). The temporal resolution of gene expression, where the down- as well as upregulation is reflected by GFP signal, requires GFP with a short half-life. Wild-type GFP is very stable with a half-life of at least 24 h in *Escherichia coli* (Bongaerts *et al*., 2002). Modifications of the C-terminal end of the GFP protein have, however, resulted in variants which are susceptible to the action of indigenous housekeeping proteases, resulting in GFP derivates with half-times as short as 40 min when synthesized in *E. coli* and *Pseudomonas putida* (Andersen *et al*., 1998).

During introduction of transcriptional fusions onto the chromosome, the location of recombination is crucial. Random integration of genes into the chromosome is often associated with uncontrolled transcriptional fusions, insertional mutations (Winson *et al*., 1998), or knock-out of downstream genes that may be essential for *in vivo* conditions. Bacterial phage systems are often exploited to transfer genes onto the bacterial chromosomes through site-specific recombination between the DNA recognition sites *attP* on the phage and *attB* on the bacterial chromosome (Groth and Calos, 2004). The discovery that the bacteriophage λ red system, plasmid- or chromosome-encoded, promotes recombination between the bacterial chromosome and linear dsDNA sequences allowed greater freedom in site-directed recombination (Murphy, 1998; Datsenko and Wanner, 2000). To decrease the risk of altering the bacterial phenotype, genetic cloning should be performed in a pseudogene or a region with no open reading frame, optimally downstream of a strong tran-

Fig. 1. Major tissue events during pyelonephritis.
A. The natural, ascending route of infection in pyelonephritis.
B. Schematic representation of a nephron, illustrating the different microenvironments bacteria must pass when ascending to the proximal tubule.
C–E. Schematic representation of tissue events occurring (C) 2–3 h post injection of bacteria into the proximal tubules; (D) 8 h post injection); (E) 22 h post injection. A detailed description of the symbols is boxed.
F–H. Visualization of tissue responses to bacterial infections in real time using multiphoton microscopy. (F) One hour, (G) 5 h and (H) 22 h post injection of GFP$^+$-producing bacteria (green) into the proximal tubule (top = x–y direction, bottom = x–z direction). Fluorescent 500 kDa dextran labels blood flow (red), 10 kDa labelled dextran outlines the injected tubule (bright blue) and proximal tubules are identified by dull green autofluorescence. Arrow in (F) indicates bacteria. Scale bar = 30 μM.

scriptional terminator. Mansson *et al*. exploited the *cobS* gene in the uropathogenic *E. coli* (UPEC) chromosome for the introduction of $P_{LtetO\text{-}1}$-*gfp*$^+$ sequence, utilizing the λ red one-step allelic replacement method (Datsenko and Wanner, 2000; Mansson *et al*., 2007). This represents a successful cloning event achieving genetic stability and constant expression of detectable fluorescent signal from a single-copy gene fusion, throughout the progression of infection inside a living animal.

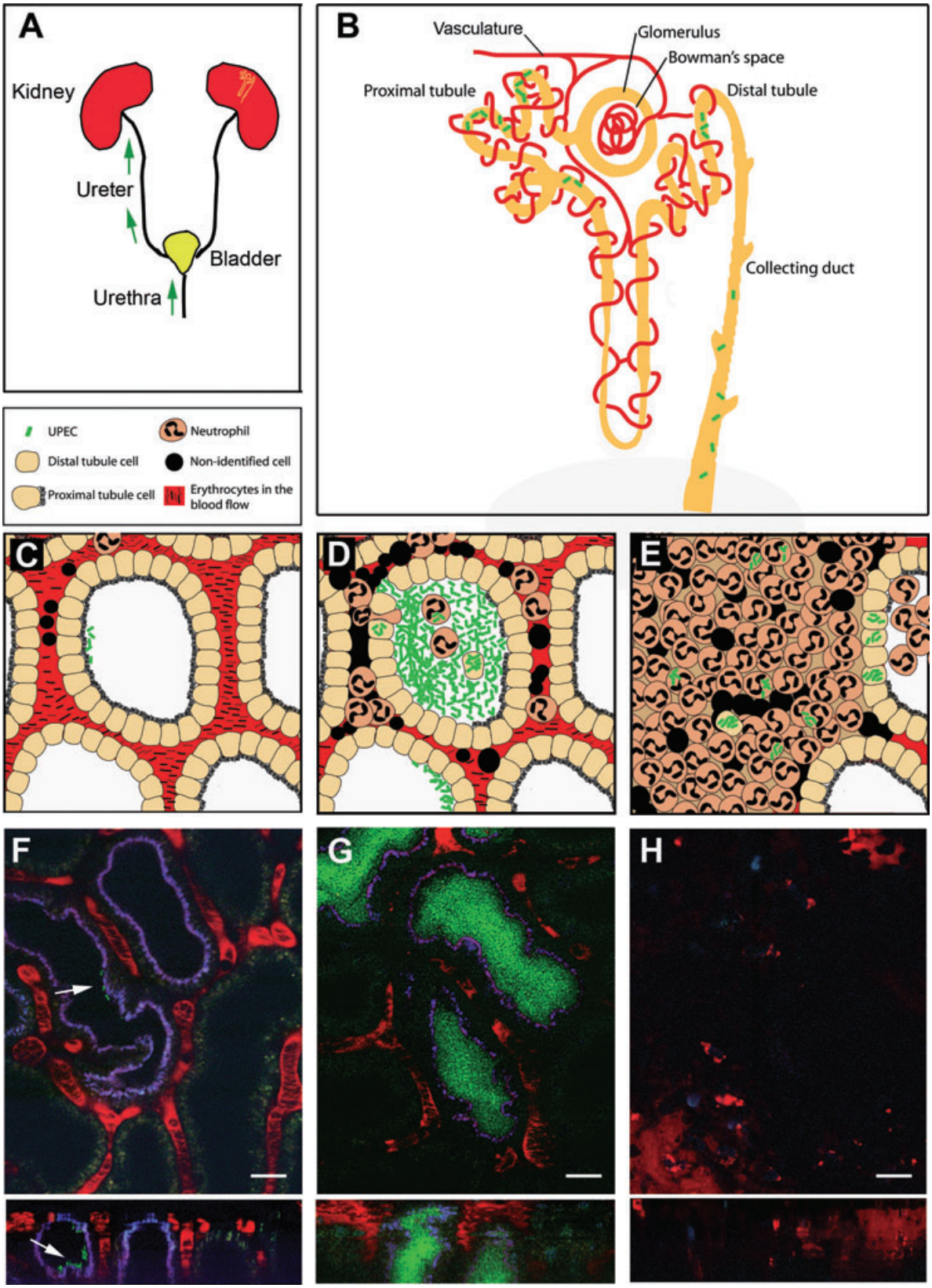
Real-time UTI – a model to study complex tissue responses to infections

The kidney is a multicellular organ which offers a clear demonstration of how bacteria can adapt to a variety of microenvironments and challenge a fully armed host immune system, and several studies have shown that UTI serves as an excellent model to study how the host immune system responds to bacterial infections (Svanborg *et al*., 2006). The kidney offers several advantages for multiphoton studies (Dunn *et al*., 2002). This organ is highly vascularized with capillaries intertwined among the nephrons. The close proximity between the vasculature and tubular epithelial cells enables simultaneous capture of the activities on the colonized epithelial lining, as well as in the blood flow. This vascularization also results in rapid delivery of the intravenous dyes and probes included in the study. Probes that are cleared from the blood by filtration in the glomerulus are made available to the apical side of the tubular epithelial cells by the glomerular filtrate. As the kidney has been the object of extensive multiphoton microscopy for physiological studies, a library of suitable dyes to visualize tissue cells as well as blood flow has already been identified (Dunn *et al*., 2002). Examples of these are the vascular delivery of fluorescent tagged dextran molecules of various sizes, which subsequently target different compartments. Large dextran molecules (500 kDa) cannot be filtered by the glomerulus and are therefore retained in the bloodstream labelling the bulk fluid phase. Small dextran molecules (10 kDa) are readily filtered and are internalized into endosomes of the proximal tubule cells. Even in the absence of dyes, the highly structured kidney is interpretable through differential autofluorescence.

During the natural retrograde route of infection, bacteria enter the urinary tract via the urethra into the bladder from where they ascend through the ureter to the kidney (Fig. 1A). Once reaching the kidney, bacteria ascend through the collecting duct into the nephron (Fig. 1B). This route of dissemination adds a degree of temporal randomness, shared among most pathogenic bacteria colonizing inner mucosal linings of the body prior to establishing infection. To enable kinetic resolution in the UTI model used by Mansson *et al*., bacteria were site-specifically delivered into the lumen of renal proximal tubules by micropuncture (Fig. 2), a well-established technique in studying renal function (Tanner *et al*., 2005). The combination of live multiphoton imaging with the spatial and temporal resolution of infection achieved by micropuncture injection created a novel animal model which has been termed ‘real-time UTI’.

Utilizing this real-time UTI model the infection process was followed from the first interaction of bacteria with the epithelial lining, through the dynamic immune response, to the end stage of massive tissue destruction (Mansson *et al*., 2007). This dynamic course of events, which is summarized in Table 2, was accomplished within 22 h, a time frame substantially shorter than previously appreciated. The co-injection of bacteria with small-molecular-weight dextran (10 kDa) labelled the injected proximal tubule revealing which nephron to study on the microscope. Subsequently all phases of bacterial colonization could be followed. Bacteria were visualized as they disseminated throughout the various microenvironments of the kidney, colonizing both proximal and distal segments of the nephron. This represents the strength of multiphoton microscopy in allowing an estimation of the significance of an observation, as an observation can be evaluated in perspective to the complete picture of infection. In contrast, confocal microscopy of tissue preparations makes it difficult to assess whether the frequency of an observation is a reflection of the tissue slide and time point chosen, or a true finding.

Systemic distribution of large-molecular-weight dextran (500 kDa) enables visualization of blood flow. Erythrocytes and white blood cells in the bloodstream exclude dextran, and are visible as black streaks, from which the rate of flow can be calculated (Molitoris and Sandoval,



2005). Within the first hours of colonization, the blood flow in the peritubular capillaries adjacent to infected tubules was shown to be dramatically altered. Capillaries became congested to the degree that erythrocytes could not pass, and in some areas blood flow was completely stopped. These effects were accompanied by increased endothelial permeability, detected as perivascular leakage of the large dextran, causing oedema in the area between the site of infection and the renal capsule. Neutrophils, identified by their distinguishing nuclear shape, were identified in the area of infection within 2–3 h and were present in large numbers after 8 h. At 22 h post infection the colonized nephron was completely shut down, with massive tissue destruction apparent at the site of infection. The above-mentioned tissue dynamics is shown as a cartoon in Fig. 1C–E, which is based on the tissue events observed in the multiphoton microscope, shown in Fig. 1F–H.

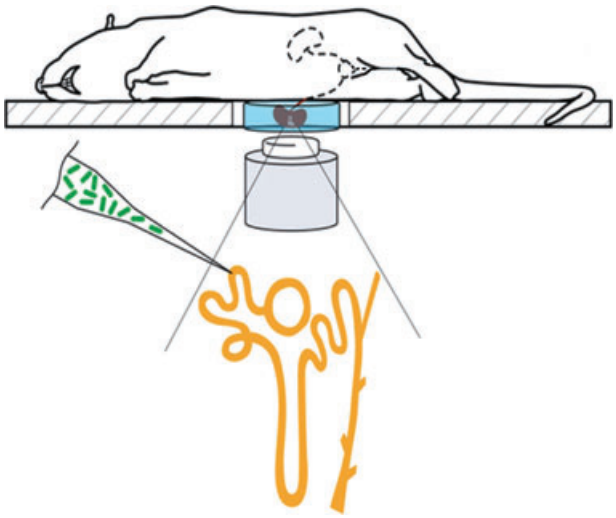


Fig. 2. Real-time UTI model. Bacteria (green) are injected by micropuncture directly into the proximal tubule of a nephron (yellow) in an exteriorized kidney. Then, the rat is immediately placed on the stage of a multiphoton microscope, with the injected kidney placed in a saline-filled glass-bottom Petri dish. Using multiphoton microscopy, the progression of infection can be visualized in the anaesthetized rat for an extended period of time.

The multiphoton microscopy technique allows a unique opportunity to study the effect of bacterial virulence factors *in vivo*, as subtle and/or transient effects on the tissue dynamics can be recorded. When investigating a role for the UPEC-associated exotoxin haemolysin (Hly), a marked delay in the immune response was seen upon infection with a Hly knockout UPEC strain in comparison with the isogenic wild-type strain (Mansson *et al.*, 2007). These data confirm observations made *in vitro*, where Hly-induced Ca^{2+} signalling caused production of pro-inflammatory cytokines in primary rat proximal tubule cells (Uhlen *et al.*, 2000). The role of Hly *in vivo* appears to modulate the dynamics of the inflammatory response rather than affecting the end result of infection, as this was shown to be independent of Hly. Future experiments will study the effects of other pyelonephritis-associated virulence factors *in vivo*, such as attachment organelles (pili, fimbriae), toxins (CNF1 and Sat) and LPS.

Limitations of the real-time UTI model

The real-time UTI model is a promising example of how multiphoton microscopy can be applied to study infectious disease. However, it must also be recognized that this is still a model, and as such is not perfect. Bacteria are prepared at present in LB broth *in vitro* and may therefore not display the same expression profile as one would expect from bacteria which have ascended through the lower urinary tract. From the current knowledge, however,

Table 2. Time resolution of events during acute pyelonephritis.

Tissue response: (time after micropuncture of proximal tubule)	Early (2–3 h)	Intermediate (5–8 h)	Late (22 h)
Proximal tubule colonization	+	+++	n/a
Multicellular bacterial communities	++	+	n/a
Bacterial filamentation	–	++	n/a
Proximal epithelia internalization	–	++	++
Disruption of epithelial lining	–	++	++++
Detachment of proximal tubular cells	–	++	+++
Distal tubule colonization	+	++	n/a
Distal epithelia internalization	–	+	n/a
Bacteria in the interstitial space	–	+	+
Endothelial internalization	–	+	n/a
Vascular dysfunction	+	+++	No flow
Neutrophil recruitment	+	++	++++
Neutrophil phagocytosis of bacteria	–	+	+++
Tubular neutrophil localization	–	++	+++
Glomerular infection	–	–	–

n/a, not applicable due to tissue destruction and bacterial clearance.

it can be seen that the bacteria adapt and colonize very quickly to their new environment as would be expected of a naturally infecting pathogen. The injection of the bacteria directly into the tubule, while allowing a large degree of spatial and temporal control, is not the natural route of infection, and the results obtained must be recognized for what they are, a model of infection. That said, the model allows a unique perspective into the tissue events occurring immediately after bacteria come into contact with host tissue, a perspective unobtainable with other methodology. The results being garnered from this model are opening up numerous new fields of investigation such as looking into the effect of infection on other physiological processes such as blood and filtrate flow, processes that are very difficult to study using other techniques.

Molecular details beyond multiphoton imaging

The ability to identify the site of infection provides a unique possibility to microdissect the infected tissue to study the molecular details of the infection, on either protein or gene expression level, *ex vivo*. As the progression of infection can be imaged until the desired stage of infection is reached, a unique control over *in vivo* parameters is obtainable. The unstable prokaryotic mRNA has proven to be difficult to isolate from infected organs, conceivably because it constitutes only a minority of the total RNA obtained from the tissue. The experimental set-up described for real-time UTI allows for prokaryotic RNA to be isolated in a high enough quantity to enable gene expression analysis of bacterial genes (Mansson *et al*., 2007). Microarray analysis can provide information about the total transcriptional profile in bacteria present in different environments (Hinton *et al*., 2004). During recent years microarrays have been utilized to investigate the expression pattern of a variety of pathogenic bacteria during *in vivo* conditions; these include *Mycobacterium tuberculosis* (Talaat *et al*., 2004), *E. coli* (Motley *et al*., 2004; Snyder *et al*., 2004), *S. enterica* (Eriksson *et al*., 2003), *Borrelia burgdorferi* (Revel *et al*., 2002) and *Yersinia pestis* (Lawson *et al*., 2006). These studies have demonstrated that pathogens utilize unique expression patterns within their hosts compared with *in vitro* conditions, highlighting the importance of *in vivo* models for understanding pathogenesis. Corresponding methods can also be applied to study the inflammatory response of eukaryotic genes at any given point of infection (Motley *et al*., 2004; Mansson *et al*., 2007).

The infected tissue can also be isolated, and further analysed *ex vivo* by other microscopy techniques (Richter-Dahlfors *et al*., 1997). The extensive range of antibodies specific for inflammatory mediators and bacterial compounds have made high-resolution confocal microscopy a powerful tool for detailed examination of specific infection stages on the protein level. Using laser capture microdissection (LCM) to isolate regions of interest, down to the single cell level, DNA, rRNA or protein can be purified and used for further *in vitro* examination (Fend and Raffeld, 2000). Expression in infected and non-infected host cells can be compared, and free living bacteria can be compared with bacteria attached to epithelial cells, embedded in microcolonies or intracellularly localized.

Concluding remarks

In conclusion, multiphoton studies provide great potential to study the dynamics of host–pathogen interaction during infectious diseases. Although this review uses upper urinary tract infections as an example, other examples of the technology exist. Chieppa *et al*. (2006) demonstrated the usability of multiphoton microscopy for the study of dendritic cell extension in the small bowel, performing experiments with DsRed-expressing *Salmonella*. This demonstrates the potential of multiphoton microscopy for studying the pathogenesis of a variety of gastrointestinal infections, e.g. *Helicobacter pylori*, *Shigella* and *Listeria.* Additionally, several physiological studies have shown the living brain to be suitable target for live imaging, but the power of multiphoton has not as yet been used to elucidate the true events of meningitis. We can accordingly expect that multiphoton microscopy will aid in shedding new light on those tissue responses that accompany a variety of infections, knowledge that may prove to be useful for the identification of novel targets to be used for future drug design.

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References

Amino, R., Thiberge, S., Martin, B., Celli, S., Shorte, S., Frischknecht, F., and Menard, R. (2006) Quantitative imaging of *Plasmodium* transmission from mosquito to mammal. *Nat Med* **12:** 220–224.

Andersen, J.B., Sternberg, C., Poulsen, L.K., Bjorn, S.P., Givskov, M., and Molin, S. (1998) New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl Environ Microbiol* **64:** 2240–2246.

Avogadri, F., Martinoli, C., Petrovska, L., Chiodoni, C., Transidico, P., Bronte, V., *et al.* (2005) Cancer immuno-

therapy based on killing of *Salmonella*-infected tumor cells. *Cancer Res* **65:** 3920–3927.

Bongaerts, R.J., Hautefort, I., Sidebotham, J.M., and Hinton, J.C. (2002) Green fluorescent protein as a marker for conditional gene expression in bacterial cells. *Methods Enzymol* **358:** 43–66.

Bousso, P., and Robey, E.A. (2004) Dynamic behavior of T cells and thymocytes in lymphoid organs as revealed by two-photon microscopy. *Immunity* **21:** 349–355.

Brown, E.B., Campbell, R.B., Tsuzuki, Y., Xu, L., Carmeliet, P., Fukumura, D., and Jain, R.K. (2001) *In vivo* measurement of gene expression, angiogenesis and physiological function in tumors using multiphoton laser scanning microscopy. *Nat Med* **7:** 864–868.

Cahalan, M.D., and Gutman, G.A. (2006) The sense of place in the immune system. *Nat Immunol* **7:** 329–332.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. (1994) Green fluorescent protein as a marker for gene expression. *Science* **263:** 802–805.

Chieppa, M., Rescigno, M., Huang, A.Y., and Germain, R.N. (2006) Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. *J Exp Med* **203:** 2841–2852.

Chudakov, D.M., Lukyanov, S., and Lukyanov, K.A. (2005) Fluorescent proteins as a toolkit for *in vivo* imaging. *Trends Biotechnol* **23:** 605–613.

Conger, J.D., and Burke, T.J. (1976) Effects of anesthetic agents on autoregulation of renal hemodynamics in the rat and dog. *Am J Physiol* **230:** 652–657.

Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* **97:** 6640–6645.

Deetjen, P., Brechtelsbauer, H., and Kramer, K. (1964) [Hemodynamics of the renal medulla. 3. Dye passage times in the external medullary zone and the renal vein: the blood circulation distribution in the kidney.] *Pflugers Archiv Gesamte Physiol Menschen Tiere* **279:** 281–293.

Denk, W., Strickler, J.H., and Webb, W.W. (1990) Two-photon laser scanning fluorescence microscopy. *Science* **248:** 73–76.

Denk, W., Delaney, K.R., Gelperin, A., Kleinfeld, D., Strowbridge, B.W., Tank, D.W., and Yuste, R. (1994) Anatomical and functional imaging of neurons using 2-photon laser scanning microscopy. *J Neurosci Methods* **54:** 151–162.

Dunn, K.W., Sandoval, R.M., Kelly, K.J., Dagher, P.C., Tanner, G.A., Atkinson, S.J., *et al.* (2002) Functional studies of the kidney of living animals using multicolor two-photon microscopy. *Am J Physiol Cell Physiol* **283:** C905–C916.

Eriksson, S., Lucchini, S., Thompson, A., Rhen, M., and Hinton, J.C. (2003) Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. *Mol Microbiol* **47:** 103–118.

Fend, F., and Raffeld, M. (2000) Laser capture microdissection in pathology. *J Clin Pathol* **53:** 666–672.

Gahan, C.G., O'Mahony, J., and Hill, C. (2001) Characterization of the *groESL* operon in *Listeria monocytogenes*: utilization of two reporter systems (*gfp* and *hly*) for evaluating *in vivo* expression. *Infect Immun* **69:** 3924–3932.

Groth, A.C., and Calos, M.P. (2004) Phage integrases: biology and applications. *J Mol Biol* **335:** 667–678.

Hansen, M.C., Palmer, R.J., Jr, Udsen, C., White, D.C., and Molin, S. (2001) Assessment of GFP fluorescence in cells of *Streptococcus gordonii* under conditions of low pH and low oxygen concentration. *Microbiology* **147:** 1383–1391.

Hautefort, I., Proenca, M.J., and Hinton, J.C. (2003) Single-copy green fluorescent protein gene fusions allow accurate measurement of *Salmonella* gene expression *in vitro* and during infection of mammalian cells. *Appl Environ Microbiol* **69:** 7480–7491.

Helmchen, F., and Denk, W. (2005) Deep tissue two-photon microscopy. *Nat Methods* **2:** 932–940.

Hinton, J.C., Hautefort, I., Eriksson, S., Thompson, A., and Rhen, M. (2004) Benefits and pitfalls of using microarrays to monitor bacterial gene expression during infection. *Curr Opin Microbiol* **7:** 277–282.

Lawson, J.N., Lyons, C.R., and Johnston, S.A. (2006) Expression profiling of *Yersinia pestis* during mouse pulmonary infection. *DNA Cell Biol* **25:** 608–616.

Mansson, L.E., Melican, K., Boekel, J., Sandoval, R.M., Hautefort, I., Tanner, G.A., *et al.* (2007) Real-time studies of the progression of bacterial infections and immediate tissue responses in live animals. *Cell Microbiol* **9:** 413–424.

Molitoris, B.A., and Sandoval, R.M. (2005) Intravital multiphoton microscopy of dynamic renal processes. *Am J Physiol Renal Physiol* **288:** F1084–F1089.

Motley, S.T., Morrow, B.J., Liu, X., Dodge, I.L., Vitiello, A., Ward, C.K., and Shaw, K.J. (2004) Simultaneous analysis of host and pathogen interactions during an in vivo infection reveals local induction of host acute phase response proteins, a novel bacterial stress response, and evidence of a host-imposed metal ion limited environment. *Cell Microbiol* **6:** 849–865.

Murphy, K.C. (1998) Use of bacteriophage lambda recombination functions to promote gene replacement in *Escherichia coli*. *J Bacteriol* **180:** 2063–2071.

Nakano, A. (2002) Spinning-disk confocal microscopy – a cutting-edge tool for imaging of membrane traffic. *Cell Struct Funct* **27:** 349–355.

Post, L.E., Arfsten, A.E., Davis, G.R., and Nomura, M. (1980) DNA sequence of the promoter region for the alpha ribosomal protein operon in *Escherichia coli*. *J Biol Chem* **255:** 4653–4659.

Qazi, S.N., Counil, E., Morrissey, J., Rees, C.E., Cockayne, A., Winzer, K., *et al.* (2001) *agr* expression precedes escape of internalized *Staphylococcus aureus* from the host endosome. *Infect Immun* **69:** 7074–7082.

Rawls, J.F., Mahowald, M.A., Goodman, A.L., Trent, C.M., and Gordon, J.I. (2007) *In vivo* imaging and genetic analysis link bacterial motility and symbiosis in the zebrafish gut. *Proc Natl Acad Sci USA* **104:** 7622–7627.

Revel, A.T., Talaat, A.M., and Norgard, M.V. (2002) DNA microarray analysis of differential gene expression in *Borrelia burgdorferi*, the Lyme disease spirochete. *Proc Natl Acad Sci USA* **99:** 1562–1567.

Richter-Dahlfors, A., Buchan, A.M., and Finlay, B.B. (1997) Murine salmonellosis studied by confocal microscopy: *Salmonella typhimurium* resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes *in vivo*. *J Exp Med* **186:** 569–580.

Rubart, M. (2004) Two-photon microscopy of cells and tissue. *Circ Res* **95:** 1154–1166.

Schenke-Layland, K., Riemann, I., Damour, O., Stock, U.A., and Konig, K. (2006) Two-photon microscopes and *in vivo* multiphoton tomographs – powerful diagnostic tools for tissue engineering and drug delivery. *Adv Drug Deliv Rev* **58:** 878–896.

Scholz, O., Thiel, A., Hillen, W., and Niederweis, M. (2000) Quantitative analysis of gene expression with an improved green fluorescent protein. *Eur J Biochem* **267:** 1565–1570.

Snyder, J.A., Haugen, B.J., Buckles, E.L., Lockatell, C.V., Johnson, D.E., Donnenberg, M.S., *et al.* (2004) Transcriptome of uropathogenic *Escherichia coli* during urinary tract infection. *Infect Immun* **72:** 6373–6381.

Southward, C.M., and Surette, M.G. (2002) The dynamic microbe: green fluorescent protein brings bacteria to light. *Mol Microbiol* **45:** 1191–1196.

Squirrell, J.M., Wokosin, D.L., White, J.G., and Bavister, B.D. (1999) Long-term two-photon fluorescence imaging of mammalian embryos without compromising viability. *Nat Biotechnol* **17:** 763–767.

Svanborg, C., Bergsten, G., Fischer, H., Godaly, G., Gustafsson, M., Karpman, D., *et al*., (2006) Uropathogenic *Escherichia coli* as a model of host–parasite interaction. *Curr Opin Microbiol* **9:** 33–39.

Svoboda, K., and Yasuda, R. (2006) Principles of two-photon excitation microscopy and its applications to neuroscience. *Neuron* **50:** 823–839.

Talaat, A.M., Lyons, R., Howard, S.T., and Johnston, S.A. (2004) The temporal expression profile of *Mycobacterium tuberculosis* infection in mice. *Proc Natl Acad Sci USA* **101:** 4602–4607.

Tanner, G.A., Sandoval, R.M., Molitoris, B.A., Bamburg, J.R., and Ashworth, S.L. (2005) Micropuncture gene delivery and intravital two-photon visualization of protein expression in rat kidney. *Am J Physiol Renal Physiol* **289:** F638–F643.

Theer, P., Hasan, M.T., and Denk, W. (2003) Two-photon imaging to a depth of 1000 microm in living brains by use of a Ti:Al2O3 regenerative amplifier. *Opt Lett* **28:** 1022–1024.

Tian, G.F., Takano, T., Lin, J.H., Wang, X., Bekar, L., and Nedergaard, M. (2006) Imaging of cortical astrocytes using 2-photon laser scanning microscopy in the intact mouse brain. *Adv Drug Deliv Rev* **58:** 773–787.

Torstensson, E., Kjäll, P., and Richter-Dahlfors, A. (2005) Chapter 2.2.6 Imaging techniques for the study of *Escherichia coli* and *Salmonella* infections. In *E. coli and Salmonella Cellular and Molecular Biology*. Böck, A., Curtiss, R., III, Kaper, J.B., Neidhardt, F.C., Nyström, T., Rudd, K.E., and Squires, C.L. (eds). Washington, DC: American Society for Microbiology Press. Available at: http://www.ecosal.org

Uhlen, P., Laestadius, A., Jahnukainen, T., Soderblom, T., Backhed, F., Celsi, G., *et al.* (2000) Alpha-haemolysin of uropathogenic *E. coli* induces Ca^{2+} oscillations in renal epithelial cells. *Nature* **405:** 694–697.

Valdivia, R.H., and Falkow, S. (1997) Fluorescence-based isolation of bacterial genes expressed within host cells. *Science* **277:** 2007–2011.

Wang, E., Babbey, C.M., and Dunn, K.W. (2005) Performance comparison between the high-speed Yokogawa spinning disc confocal system and single-point scanning confocal systems. *J Microsc* **218:** 148–159.

Wiles, S., Hanage, W.P., Frankel, G., and Robertson, B. (2006) Modelling infectious disease – time to think outside the box? *Nat Rev Microbiol* **4:** 307–312.

Winson, M.K., Swift, S., Hill, P.J., Sims, C.M., Griesmayr, G., Bycroft, B.W., *et al.* (1998) Engineering the *luxCDABE* genes from *Photorhabdus luminescens* to provide a bioluminescent reporter for constitutive and promoter probe plasmids and mini-Tn*5* constructs. *FEMS Microbiol Lett* **163:** 193–202.

