Non-invasive three-dimensional imaging of *Escherichia coli* K1 infection using Diffuse Light Imaging Tomography combined with Micro-Computed Tomography

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Abstract

In contrast to two-dimensional bioluminescence imaging, three dimensional diffuse light imaging tomography with integrated micro-computed tomography (DLIT-μCT) has the potential to realise spatial variations in infection patterns when imaging experimental animals dosed with derivatives of virulent bacteria carrying bioluminescent reporter genes such as the *lux* operon from the bacterium *Photorhabdus luminescens*. The method provides an opportunity to precisely localise the bacterial infection sites within the animal and enables the generation of four-dimensional movies of the infection cycle. Here, we describe the use of the PerkinElmer IVIS SpectrumCT *in vivo* imaging system to investigate progression of lethal systemic infection in neonatal rats following colonisation of the gastrointestinal tract with the neonatal pathogen *Escherichia coli* K1. We confirm previous observations that these bacteria stably colonise the colon and small intestine following feeding of the infectious dose from a micropipette; invading bacteria migrate across the gut epithelium into the blood circulation and establish foci of infection in major organs, including the brain. DLIT-μCT revealed novel multiple sites of colonisation within the alimentary canal, including the tongue, oesophagus and stomach, with penetration of the non-keratinised oesophageal epithelial surface, providing strong evidence of a further major site for bacterial dissemination. We highlight technical issues associated with imaging of infections in new born rat pups and show that the whole-body and organ bioburden correlates with disease severity.

Keywords: bioluminescence imaging; *Escherichia coli* K1; neonatal systemic infection; gastrointestinal colonisation; neonatal meningitis; age-dependent susceptibility to infection

1. Introduction

Escherichia coli and *Streptococcus agalactiae* (the Group B streptococcus) are the leading causes of systemic life-threatening bacterial infections in the new born infant, especially among premature, low-birth-weight neonates [1, 2]. These opportunistic pathogens colonize mucosal surfaces after acquisition at or soon after birth and in a minority of cases breach the mucosal barrier to engender a rapidly progressive infection which the vulnerable infant is ill-equipped to counter. The large majority of *E. coli* strains causing neonatal systemic infections express the K1 capsule, a homopolymer of α-2,8-linked *N*-acetylneuraminic acid that constitutes the outermost layer of the cell and protects the bacteria from the host's immune clearance mechanisms [3]. *E. coli* K1 is an adept colonizer of the neonatal gastrointestinal (GI) tract from where it may translocate into the systemic circulation (sometimes causing an unwanted immune response, termed sepsis), enter the central nervous system and gain access to the meninges, producing meningitis [4, 5]. *E. coli* K1 systemic infections display a strong age-dependency: the bacteria are benign, common constituents of the adult GI microbiota [6] but are potentially lethal during the first weeks of life [1, 7].

Neonatal bacterial infections are medical emergencies and it is consequently difficult to obtain insights into the underlying pathogenic mechanisms of the invading bacteria from human studies alone. Importantly, key features of *E. coli* K1 systemic infections, including the age dependency, can be replicated in the neonatal rat by oral administration of bacteria, a procedure that frequently initiates stable colonization of the GI tract [8, 9]. Rat pups colonized in the first few days *postpartum* develop lethal systemic infection as the colonizing *E. coli* K1 translocate from the lumen of the GI tract to the blood circulation to cause a persistent bacteraemia [9, 10]; they then elicit infection in multiple organs, including the brain [11, 12]. Bacteria appear to enter the cerebrospinal compartment predominantly at the choroid plexus and penetrate superficial brain tissue [11], where they give rise to localized inflammation through cytokine-induced pathways [13]. A week after birth, the rats become refractory to systemic infection, even though *E. coli* K1 GI colonization may persist beyond this time point [9] due to maturation of the protective mucus layer lining the GI tract [14].

Most studies with the rodent model have relied upon culling of infected animals at various stages after initiation of colonization with *E. coli* K1 and the data provide only a "snapshot" of disease progression with time. The presence of the pathogen in selected tissues is generally revealed by viable counting, or by histological or histochemical staining of tissue sections. Real-time non-invasive

imaging in the whole organism has the capacity to reveal complex patterns of dissemination from colonization sites and the dynamic process of infection over time in individual animals. The optical imaging of bioluminescent signals from recombinant pathogenic bacteria expressing luciferase such as that provided by the *lux*CDABE operon from the terrestrial bacterium *Photorhabdus luminescens*, in combination with *ex vivo* organ analysis, has been widely adopted to furnish fresh insights into processes of infectious disease [15-18]. With conventional 2D bioluminescence imaging, the location of the signal is inferred from its location at the animal surface; a quantitative 3D reconstruction of the source of the signal may be determined by diffuse light imaging tomography (DLIT) [19]. Unambiguous non-invasive localization of bioluminescent signals from small animals without the need for organ recovery and *ex vivo* quantification can at present only be achieved using multi-modality imaging. Here, we review our recent use of a combination of DLIT co-registered with integrated micro-computed tomography (μCT) imaging to probe the infection dynamics of infection-susceptible neonatal rat pups colonized with a bioluminescent derivative of *E. coli* K1 strain A192PP. We discuss the technical challenges posed by these procedures and highlight the advantages and limitations of the technique.

2. DLIT-μCT imaging: theory and practice

Diffuse light imaging tomography utilizes a charge-coupled device (CCD) camera and band-pass emission filters to obtain a series of multi-spectral 2D images of bioluminescent sources within a living subject. The DLIT algorithms also require a measurement of the subject surface topography. Here the μCT scan serves a dual purpose: the air-tissue boundary aids in surface topography generation and provides integrated anatomical co-registration with the optical source(s).

2.1. DLIT theory

Diffuse tomographic analysis can be a rigorous approach to provide source depth and intensity information, sometimes involving complicated multi-view instrumentation [20-22] or *a priori* knowledge of allowable source distribution [23, 24]. To reduce both imaging and computational time, PerkinElmer's IVIS instrumentation uses multispectral 2D image acquisitions and diffuse luminescence tomographic algorithms [25, 26]. Tissue absorption of light across the 500-750 nm spectrum, mainly by haemoglobin, is significantly decreased at the red end of the spectrum compared to the blue/green end of the spectrum [27]. Therefore, the light output of an *in vivo* bioluminescent

source depends on the depth of tissue it must pass through, as well as its wavelength. The DLIT algorithms exploit this property; in 2D, shallow sources appear brighter than deep sources at the blue/green end of the spectrum, while deep sources appear brighter than shallow sources at the yellow/red end of the spectrum. The DLIT algorithms produce a 3D reconstruction of the bioluminescent source(s) using a non-negative least squares optimization.

DLIT also requires a measurement of the surface topography of the imaging subject in order to convert exhibited light emission into a photon density map. In the instance of DLIT- µCT, the surface topography is derived from the µCT scan itself by delineation of the air-tissue boundary. The surface topography, when combined with the series of filtered 2D bioluminescence images, results in an improved resolution of source parameters by providing uniqueness to the algorithms.

2.2. DLIT- µCT in practice

PerkinElmer's IVIS SpectrumCT includes all hardware and software required for DLIT- µCT. The system consists of a cooled (-90'C), integrating CCD camera that is placed above a light-sealed imaging chamber (Fig. 1). The heated imaging stage is mobile along the optical axis to adjust field of view and the emission filter wheel contains 18 filters with 20 nm bandwidths. In order to keep a manageable system footprint, the x-ray source and complementary metal oxide semiconductor detector are fixed perpendicular to the optical axis, while the µCT projections are captured by rotating the subject on a unique turntable arrangement (Fig. 1). DLIT- µCT acquisition consists of a µCT scan followed by a sequence of multispectral 2D bioluminescence images. Image acquisition is performed using PerkinElmer's Living Image® software. A standard single animal µCT scan was employed to provide a quality volume for co-registration while also keeping the x-ray dose low (53 mGy).

Bioluminescence images are acquired with various emission filters in place and typically 4-6 emission filters are chosen that cover the known spectrum of the bioluminescent probe of interest. In the study described here, 540, 560, 580, 600 and 620 nm filters were chosen for *lux* bacterial luciferase. The auto-exposure feature was used; this allows the software to determine the acquisition parameters needed to reach a target count of 10,000 for each 2D BLI image. Auto-exposure range parameters were adjusted to ensure maximum sensitivity: the maximum exposure time was increased to 5 min and the maximum bin was increased to 16. Whilst the most recent version of Living Image software is capable of performing 'one-click' reconstructions, the order of operations remains the same for the traditional method of reconstruction. Firstly, the subject's surface topography is

computed following the determination of the air-tissue boundary threshold of the µCT scan. Secondly, the bioluminescent images are examined for quality and threshold levels. Images that are deemed to be of insufficient quality, for example too low a bioluminescent signal, are automatically excluded from the reconstruction algorithm, but the end-user may adjust if appropriate. After the reconstruction is complete, the software automatically co-registers the reconstructed bioluminescent source(s) with the µCT scan. Thresholds can be applied to the µCT volume in order to highlight specific anatomical features of the subject.

One of the more common troubleshooting areas of *lux* DLIT- µCT is bioluminescence signal strength. The *lux* emission spectrum, with a peak at around 480 nm and only a small percentage beyond 600 nm, falls in an area of the visible spectrum that is more heavily absorbed by haemoglobin, so depending on the animal model, the starting signal strength may be low. This issue is exacerbated when the DLIT images are acquired, as each image is collecting a narrow range (20 nm) of emitted light. Thus it is imperative that all signal impeders be removed, including animal fur, and pigmented animals are best avoided. Further, it is best practice to place the subjects on the imaging stage such that the brightest side is facing the CCD camera (i.e. facing up). Finally, as mentioned above, adjusting auto-exposure parameters such that the most sensitive settings are allowed will help ensure sufficient signal levels.

3. Rodent model of neonatal *E. coli* K1 infection

All animal experiments described here conformed to national (U.K. Animals [Scientific Procedures] Act, 1986) and European (EU Directive 2010/63/EU for animal experiments) legislation and were approved by the Ethical Committee of the University College London and the UK Home Office (HO). All animal work was conducted under HO project licences PPL 80/2243 and PPL 70/7773.

The *E. coli* K1 capsule is associated with a limited number of O-serotypes, including O1, O2, O7, O18, O25 and O75, that are commonly found as causative agents of neonatal systemic infection [6, 28]. Many of these isolates readily colonize the GI tract when administered orally to neonatal rat pups, with colonization rates of 88-100%, but they vary in their capacity to cause bacteraemia (2-50%) and to induce lethality (0-25%) [9]. Clones belonging to serotype O18 tend to cause the most severe systemic neonatal infections in rodents [9]. In order to undertake extensive characterization of the

basis of systemic infection due to *E. coli* K1, we selected the O18:K1 septicaemia isolate A192 [28], reported as colonizing new born Wistar rats with an incidence of 100%, inducing bacteraemia in 35% with survival of 75% [12]. This strain, like many other *E. coli* K1 strains, belongs to sequence type 95 [29]. We enhanced the virulence of *E. coli* A192 by two rounds of passage through susceptible neonatal rats to obtain a derivative, *E. coli* A192PP, that produced lethal disseminated infection in all colonized two-day-old (P2) animals [10, 11]. The substantial enhancement of virulence was due to only four single nucleotide polymorphisms, each within genes involved in metabolic functions [29].

Feeding of 2-6 x 10^6 CFU mid-logarithmic *E. coli* K1 to P2 Wistar rat pups through an Eppendorf pipette established a rapidly progressive lethal infection; as the pups matured over a seven day period, they became progressively less susceptible to bacteraemia and to the lethal effect (Fig. 2), but not to GI colonization [30, 31]. For DLIT-μCT imaging, we engineered *E. coli* A192PP for expression of the bioluminescence phenotype by introduction of the *lux* operon through mini-Tn*5* mutagenesis [12]. It is imperative to ensure that bioluminescent conjugants produce a strong bioluminescent signal over the course of the growth cycle in the absence of the selective antibiotic to confirm constitutive and stable expression of the *lux* operon [32], display a rate of growth in nutrient-replete medium comparable to that of the parent strain and retain a comparable level of virulence in the animal model. We investigated over thirty bioluminescent derivatives of *E. coli* A192PP and identified only one suitable *lux* strain for detailed *in vivo* investigations, *E. coli* A192PP-*lux2* (Fig. 3), which showed only a small reduction in virulence compared to the parent [12]. Thus, three-dimensional DLIT-μCT, in combination with enumeration by culture of organ bioburden and two-dimensional bioluminescent imaging, was employed to illustrate temporal and spatial aspects of systemic infection in P2 rat pups following oral administration of *E. coli* A192PP-*lux2* [12]. Bacteria translocated from the lumen of the GI tract to the blood compartment and entered the central nervous system with colonisation of the brain restricted to the meningeal surfaces. DLIT-μCT demonstrated for the first time the colonisation of additional sites from the oral bolus; these included keratinised and non-keratinised surfaces of the oesophagus and the tongue. The total *E. coli* K1 burden increased with time and was highly correlated with severity of symptoms.

3.1. Preparation and use of neonatal rat pups for DLIT-μCT imaging of infection

Neonatal rats require immobilisation for up to forty minutes in order to achieve high quality DLIT-μCT images. We have noted, as have others [33, 34], that very young neonatal rat pups do not respond

favourably to anaesthetic drugs such as isoflurane and it has been recorded that prolonged exposure of the developing rat brain to commonly used general anaesthetics triggers widespread regional apoptotic neurodegeneration [34-36] and increases blood-brain barrier permeability [37], which is likely to influence the outcome of neurotropic *E. coli* K1 infections. Nevertheless, it was not possible to immobilise P2 pups for sufficient time without sedation and it proved equally difficult to constrain the infection-resistant P9 pups that we have used extensively in our studies of age-related susceptibility to infection. We subsequently determined that sufficient sedation of P2 pups could be induced with 5% isoflurane followed by maintenance under 2.5% isoflurane [12]. Pups were culled after 3D DLIT-μCT imaging; we deemed such destructive sampling necessary as repeated or prolonged induction of anaesthesia with isoflurane increases the risk of maternal rejection [38], neonatal rats are more prone to post-anaesthesia complications than adults [39] and anaesthesia increases the risk of progression of a number of non-infectious conditions [40, 41]. We cannot exclude the possibility that similar modifications may be relevant to bacterial infectious disease.

Thus, progression of infection was monitored with DLIT-μCT by selection of pups colonised with *E. coli* K1 at various times after oral dosing of the pathogen at P2 and by adopting a multi-imaging approach [12]. DLIT-μCT was combined with 2D bioluminescent imaging (2DBLI) by both destructive and longitudinal sampling and enumeration of organ bioburden by traditional viable counting techniques, to obtain a comprehensive picture of dissemination, replication and invasion of the pathogen (Fig. 4). As expected, whole-body and organ bioburden correlated with disease severity. 2D images could be obtained relatively quickly without sedation and pups were sufficiently immobilised using black cloth supports placed either side and sometimes behind the pup once the animal had settled in the heated imaging chamber. For longitudinal studies, pups were rubbed against their littermates and bedding to prevent maternal rejection and then labelled with a non-scented marker pen suitable for skin application. DLIT-μCT videos of infected P2 pups can be viewed by following the links in the supplemental files for reference [12]: http://iai.asm.org/content/83/12/4528/suppl/DCSupplemental.

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Figure legends

Figure 1. Schematic of the IVIS SpectrumCT – an advanced 3D combined optical and low dose µCT imaging system, allowing the simultaneous collection of molecular and anatomical data both non-invasively and longitudinally from experimental mice and rats. The automated gantry moves vertically to allow different fields of view for the collection of optical images (grayscale photos) and data (e.g., bioluminescence), while the flat panel detector and X-ray source of the µCT rotate horizontally to acquire full 3D X-ray data sets.

Figure 2. Age dependency of systemic infection following oral administration of *E. coli* A192PP to neonatal rat pups (*n*=24 for each group) aged from two days (P2) to nine days (P9). Adapted from Dalgakiran *et al* [23].

Figure 3. Stable constitutive bioluminescence of *E. coli* A192PP-*lux2* and *E. coli* A192PP-*lux12*. The former conjugant was selected for imaging of *E. coli* K1 systemic infection due to retention of virulence in the neonatal rat and superior bioluminescence properties. (A) 2DBLI of A192PP-*lux2* (tubes 1 & 2), A192PP-*lux12* (tubes 3 & 4) and *Citrobacter rodentium* strain ICC180 (employed as described in ref 24) for comparison. (B) Mueller-Hinton agar cultures of (top) of A192PP-*lux2* and A192PP-*lux12*; (bottom) A192PP wild type and *C. rodentium* ICC180; visualized by 2DBLI. Images of colonies of A192PP-*lux2* (C) and A192PP-*lux12* (D) obtained using the Bio-Rad ChemiDoc bioluminescence imaging system.

Figure 4. Dissemination of *E. coli* A192PP-*lux*2 to regional lymph nodes in live animals and excised nodes 72 h after oral application of 2-6 X 10^6 CFU bacteria, revealed by 2DBLI and DLIT-µCT. Arrows indicate excised tissues. Images were collected from live animals; they were then sacrificed, organs collected and images obtained immediately. Representative images of whole animals and excised organs are shown; different animals were used to generate each image. [Infection and Immunity 2015; **83,** 4528-4540. doi: 10.1128/IAI.00953-15].

Appendix 1

The equipment needed to conduct DLIT-μCT investigations with small animals is available commercially. Details of equipment and supply lists, and general data relevant to the technique is available from PerkinElmer: http://www.perkinelmer.com/product/ivis-instrument-spectrum-ct-120v-128201